

TEMPERATURE REGULATION OF SHIGELLA VIRULENCE:
IDENTIFICATION OF TEMPERATURE-REGULATED SHIGELLA
INVASION GENES BY THE ISOLATION OF inv:lacZ OPERON
FUSIONS AND THE CHARACTERIZATION OF THE VIRULENCE
GENE REGULATOR virR

1991

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ABSTRACT

Title of Dissertation:

Temperature Regulation of *Shigella* Virulence: Identification of Temperature-Regulated *Shigella* Invasion Genes by the Isolation of *inv::lacZ* Operon Fusions and Characterization of the Virulence Gene Regulator *virR*.

Alexander E. Hromockyj; Candidate, Doctor of Philosophy, 1991

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Penetration and multiplication within cells of the human colonic epithelium are hallmarks of *Shigella* spp. pathogenicity. A feature of *Shigella* spp. virulence is that it is regulated by growth temperature. Strains are phenotypically virulent when cultured at 37°C, but are phenotypically avirulent when cultured at 30°C. Temperature-regulated virulence genes involved in *Shigella* spp. pathogenesis and the regulator of the temperature-regulated virulence phenotype, *virR*, have been identified. The number of genes involved in *Shigella* spp. pathogenicity and the number of virulence genes that are temperature-regulated is unknown. The nature of the virulence gene regulator, *virR*, also remains to be determined. In these studies temperature-regulation of *Shigella*

spp. virulence was approached from two perspectives: first, from the aspect of the genes regulated in response to environmental temperature changes, and second from the aspect of the temperature-dependent virulence gene repressor *virR*.

lacZ operon fusion technology was employed to identify temperature-regulated virulence genes in *Shigella flexneri* serotype 2a. Four operon fusions identified in temperature-regulated invasion genes (*inv::lacZ*) were found to be: i) unable to invade HeLa cells; ii) located in a region of the 220 kb invasion plasmid defined as the minimal amount of DNA required for invasion; and, iii) controlled by *virR*. Western blot and Southern hybridization analysis indicated that three of the fusions were within genes mapping to regions previously identified as essential for a positive virulence phenotype. Analysis of bacterial surface proteins suggested that the genes marked by these fusions play a role in the correct surface expression of the *ipaB* and *ipaC* gene products. These genes were designated *mxi* (membrane expression of Ipa). A fourth fusion was localized to a known *inv* gene, *ipaB*, which encodes one of the major immunogenic peptides of *Shigella* spp. This *ipaB::lacZ* operon fusion mutant synthesized a truncated IpaB protein recognized by IpaB-specific monoclonal antibodies. The truncated product was detected on the surface of the mutant strain by whole cell ELISA. One of the temperature-regulated *mxi* gene promoters was also isolated and shown to require a virulence plasmid-encoded transcriptional activator for activity.

Virulence in both *Shigella* spp. and enteroinvasive *Escherichia coli* strains is growth temperature regulated. *Shigella* and *E. coli* share >90% DNA sequence homology. Nonpathogenic *E. coli* K-12 strains, C600 and

MG1655, were used to determine if laboratory *E. coli* strains contain a homologous *virR* gene. Bacteriophage Plvir transduction of either C600 or MG1655 DNA into a mutant *virR Shigella* recipient resulted in complementation of the mutation. The *virR* homolog was cloned from C600 using cosmid vector pCVD301 and shown to complement a *virR* defect and restore temperature-regulated expression in a *Shigella inv::lacZ* operon fusion mutant. It was concluded that *E. coli* K-12 does harbor a gene functionally homologous to *virR* and that the *virR* homolog may serve as a global regulator of genes other than those involved in virulence.

The *virR* gene of *Shigella* was subcloned from the previously isolated *virR* cosmid clone pATM003. *VirR*⁺ clones were identified by complementation of a Δ *virR* mutation and restoration of temperature-regulated β -galactosidase expression from an *inv::lacZ* operon fusion. The *virR* locus was mapped to a 1.8 kb *EcoRI*-*AccI* restriction fragment by Southern hybridization analysis. Nucleotide sequence analysis of the 1.8 kb fragment revealed that the complete coding sequence of the *E. coli hns* gene was encoded on this fragment. Insertional mutagenesis of this coding sequence caused a loss of *VirR*⁺ activity. It was concluded that the *S. flexneri virR* gene is an allele of *hns*, the gene which encodes the histone-like cold-shock protein (H-NS). An additional clone was found to express a *VirR*^P phenotype or an incomplete repression of reporter gene transcription at 30°C. Southern hybridization, nucleotide sequence and mapping analyses of the *VirR*^P clone indicated that it was distinct from the *virR* clone and established that the *VirR*^P phenotype was due to multiple copies of the tyrosine transfer RNA (tRNA^{Tyr}) gene *tyrT*. Models for the temperature-dependent *hns* and *tyrT* regulatory mechanisms are proposed.

Temperature Regulation of *Shigella* Virulence:
Identification of Temperature-regulated *Shigella* Invasion Genes
by the Isolation of *inv::lacZ* Operon Fusions
and
Characterization of the Virulence Gene Regulator *virR*.

by
Alexander Eugene Hromockyj

Dissertation submitted to
the Faculty of the Department of Microbiology
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requirements for the degree of
Doctor of Philosophy 1991

DEDICATION

I dedicate this dissertation to my parents and my wife. My parents came to this country during difficult times and through hard work and determination they persevered to raise me in an environment where I learned to believe that anything is possible as long as you try. They always believed in me. To my wife Sophie who has made the last two years of my life the very best.

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INTRODUCTION

Significance of bacterial virulence gene regulation.

Bacterial pathogens frequently find themselves exposed to various hostile conditions during their infectious cycle. These organisms have evolved to adapt and thus survive the growth conditions encountered both inside and outside of their respective hosts. An organism's adaptive response involves the ability to synthesize proteins which facilitate their survival under different growth conditions. A bacterial pathogen's ability to establish and maintain itself within a host is facilitated by the synthesis of proteins encoded by a subset of genes referred to as virulence genes. In addition to protein synthesis, the adaptive response also involves mechanisms for sensing environmental change and in turn regulating gene expression. Consequently, protein synthesis required during varied growth conditions can be modulated accordingly. Many examples exist of non-pathogenic bacteria regulating different genes and operons in response to changes in their surroundings. A shift in growth from one carbon source to another, nutrient limitation in the form of inorganic phosphate or fixed nitrogen, a change from aerobic to anaerobic growth conditions, and changes in environmental osmolarity and temperature are all signals to which bacteria can generate an adaptive response by alteration of gene expression (reviewed by Gottesman, 1984; Stock *et al.*, 1989). Recently, evidence has accumulated which demonstrates that bacterial pathogens are capable of regulating virulence gene expression in response to many of the environmental signals noted above which are encountered during their infectious cycle (reviewed by Miller *et al.*,

1989; DiRita and Mekalanos, 1989). Studies to elucidate the mechanisms of gene regulation in response to environmental change indicate that bacteria have integrated different regulatory circuits into global regulatory systems or regulons, whereby genes and operons physically scattered throughout the genome are controlled by a central regulator (Gottesman, 1984).

The evolution of mechanisms for the regulation of bacterial virulence genes and their organization into global regulatory systems appears to be advantageous for the pathogenic bacterium in two ways. First, the organism conserves energy by repressing the expression of certain genes when the gene products are not needed and, second, the organism has an efficient means of coordinately regulating the expression of unlinked genes in response to an environmental stimulus. Bacterial pathogens therefore, are highly specialized organisms capable of monitoring and adapting to changes in their surroundings through economical expression of a complex network of genes and operons which are necessary for their survival.

Significance of temperature-regulated virulence gene expression in *Shigella*.

For human pathogens, temperature is an obvious environmental cue which could be exploited to trigger the regulation of virulence genes. The temperature within the host is generally 37°C, while outside of the host, the temperature is <37°C in most cases. Thus, a shift from 30°C to 37°C could indicate to the bacteria that virulence genes should be turned on. The human enteric pathogen *Shigella*, appears to use this change in

temperature as a signal to regulate the expression of a number of virulence genes (Table 1; reviewed by Maurelli, 1989a). Although *Shigella* is not unique in regulating virulence gene expression in response to temperature (Maurelli, 1989b), the molecular mechanism of temperature-regulated *Shigella* virulence gene expression may serve as a model system of gene regulation in response to temperature.

Epidemiology and pathogenesis of bacillary dysentery.

Bacillary dysentery (shigellosis) has been associated with significant mortality throughout history (Formal and Levine, 1983). The disease is responsible for causing 10-20% of acute diarrheal disease worldwide (Kopecko *et al.*, 1985). Although endemic throughout the world, the advent of more efficient means of sanitation and a greater degree of personal hygiene has made shigellosis less prevalent in western, industrialized countries. Shigellosis, however, remains of special concern in developing nations where it is significantly associated with malnutrition and is an important contributing cause of death in children up to the age of 6 years (Keusch and Formal, 1984). Bacillary dysentery is exclusively a disease of man and higher primates and is primarily caused by the enteric pathogen *Shigella* and to a lesser extent by the genetically closely related enteroinvasive strains of *Escherichia coli* (EIEC; Kopecko *et al.*, 1985). The genus *Shigella* consists of four serotypes. *S. flexneri* is the most prevalent cause of disease in developing countries, while *S. sonnei* and *S. boydii* are occasionally isolated. *S. dysenteriae* occurs in epidemic outbreaks and causes the most severe disease.

TABLE 1. Phenotypes associated with *Shigella* virulence^a.

Virulence-associated phenotype	Regulation by growth temperature	Reference
Maintenance of 220 kb virulence plasmid	No	Maurelli <i>et al.</i> , 1984b
Invasion of cultured mammalian cells	Yes	Maurelli <i>et al.</i> , 1984b
Invasion and intracellular multiplication	Yes	Oaks <i>et al.</i> , 1985
Invasion and keratoconjunctivitis production in guinea pig (Sereny test)	Yes	Maurelli <i>et al.</i> , 1984b
Contact hemolytic activity	Yes	Clerc <i>et al.</i> , 1986
Pigmentation on Congo red agar	Yes	Maurelli <i>et al.</i> , 1984b
Aerobactin production	No	(unpublished)
Shiga toxin production (<i>S. dysenteriae</i> 1)	Yes	Weinstein <i>et al.</i> , 1988
<i>ipa</i> gene product expression		Maurelli <i>et al.</i> , 1985
IpaA (78.kDa)	Yes	
IpaB (62.1 kDa)	Yes	
IpaC (43 kDa)	Yes	
IpaD (37 kDa)	Yes	
<i>mxi</i> gene expression		Hromockyj and Maurelli, 1989
<i>mxiA</i>	Yes	
<i>mxiB</i>	Yes	

^a From Maurelli, 1989a

Shigellosis results from ingestion of the pathogen in contaminated food or water. The ingestion of as few as ten organisms can initiate full clinical disease (Levine *et al.* 1973). This distinguishes *Shigella* from EIEC which require an infectious dose of over 10^8 organisms to cause an infection (Dupont *et al.*, 1971). The low infectious dose suggests that the organisms are somewhat resistant to the gastric acid barrier of the stomach (Formal and Levine, 1983). The primary site of infection is the colon. Pathogenicity of *Shigella* spp. is characterized by the ability of the organisms to invade and multiply within the mucosal epithelial cells of the colon (LaBrec *et al.*, 1964). However, prior to reaching the epithelial cell the bacteria must penetrate the mucous and glycocalyx layers which coat the epithelial cells (Formal and Levine, 1983). Electron micrographic studies of the events following bacterial contact with the epithelial cells (Takeuchi *et al.*, 1965) indicate a general pattern of steps which define the invasive process (Fig. 1). These studies revealed that there is an initial localized destruction of the brush border of the intestinal epithelium followed by epithelial cell engulfment of the organisms in a process referred to as induced phagocytosis. The organisms reside temporarily within a cytoplasmic vacuole made up of the host cell membrane and are next observed free in the cytoplasm. Bacteria free within the cytoplasm multiply rapidly and spread laterally from the initially infected cells to adjacent epithelial cells. The infection is generally confined to the superficial layers of the intestinal mucosa. Bacteria reaching the lamina propria evoke an intense inflammatory reaction and are efficiently killed. The successive invasion and intercellular spread however, lead to epithelial cell death

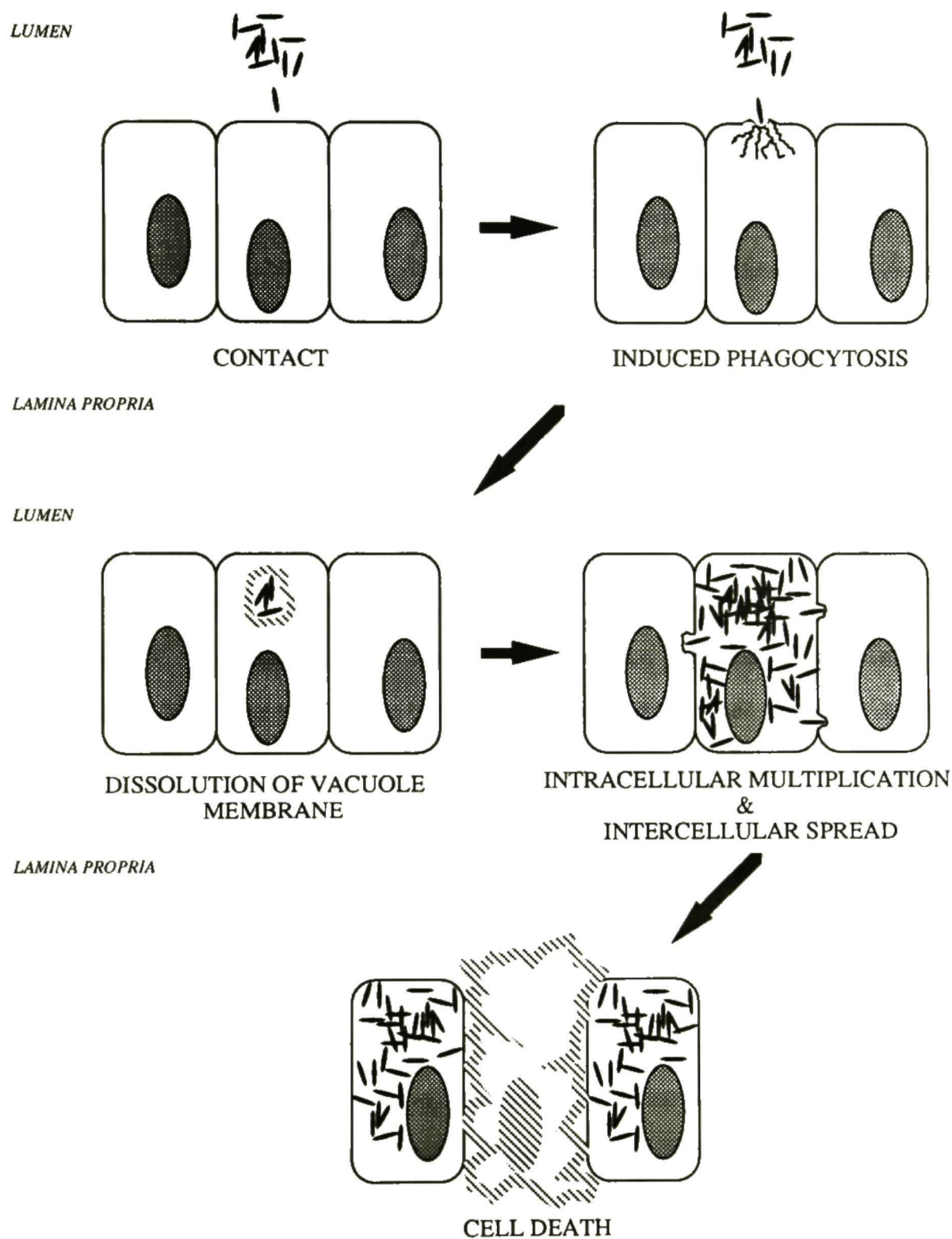


FIGURE 1. Proposed steps in *Shigella* invasion. Steps of invasion are shown after the bacteria have gained access to the lumen of the colon.

and inflammation which in turn lead to a more severe inflammatory reaction which results in the formation of ulcerative lesions in the mucosal epithelium and fluid secretion into the lumen of the colon (Takeuchi *et al.*, 1965). Ultimately, this invasive process results in disease symptoms which can range from a mild diarrhea to a severe dysenteric syndrome with blood, mucus, and pus in the stools. The disease is generally self-limiting, but in extreme cases, it can progress to a state of hypotensive shock and result in death (Formal and Levine, 1983).

Assays for measuring *Shigella* virulence.

As mentioned previously, shigellosis is exclusively a disease of man and higher primates. The innate resistance of most animals to oral challenge by *Shigella* species has complicated the ability to easily measure the virulence potential of different strains. Although the monkey model is the assay of choice to determine virulence potential, several inexpensive small animal and tissue culture models have been developed to study the pathogenesis of *Shigella* infections in greater detail. The assays used in this study are described below.

Semi-confluent monolayers of cultured mammalian cells of epithelial and non-epithelial origin (*e.g.*, Henle and HeLa cells) are commonly used as a model of the intestinal epithelium (Hale and Formal, 1981). Virulent strains of *Shigella* are capable of invading these cells through an induced phagocytosis, a process which is similar to that of phagocytosis by professional phagocytes. It involves actin polymerization and myosin accumulation (Clerc and Sansonetti, 1987) and appears to mimic the first step in intestinal epithelial cell invasion described previously (Takeuchi

et al., 1965). Internalized bacteria initially reside within a cytoplasmic vacuole before being detected free within the cytoplasm. Thus, escape from the phagocytic vacuole is a necessary step for spread of the invading organism to adjacent cells. Electron micrographic studies of invaded HeLa cell monolayers have shown that fully virulent bacteria are free within the cytoplasm by 30 minutes after invasion (Sansonetti *et al.*, 1986). These same studies showed a direct correlation between the ability of virulent organisms to escape from the phagocytic vacuole and their ability to express a bacteria-bound hemolysin. Concurrently, an assay measuring the contact-mediated hemolytic potential of virulent *Shigella* strains was developed (Clerc *et al.*, 1986).

Bacteria free within the target cell cytoplasm multiply rapidly and then spread to contiguous cells. Quantitation of the capacity to replicate intracellularly and spread intercellularly is accomplished using the plaque assay (Oaks *et al.*, 1985), a modification of the tissue culture invasion model mentioned above. Confluent monolayers of mammalian cells are challenged with virulent organisms and invasion and intercellular spread result in a cytopathic effect which is detected as a clearing of the monolayer or plaque.

The *in vitro* models, however, do not measure the ability of virulent organisms to survive host defense mechanisms which are encountered during a natural infection. This is accomplished through the use of animal models such as the Séreny test (Séreny, 1955). The Séreny test assesses the ability of virulent shigellae to invade the corneal epithelium of a guinea pig and to elicit a keratoconjunctivitis 24 to 48 hours post-inoculation. Invasion and persistence of the organism in the corneal

epithelium results in destruction of the mucosal surface and an inflammatory response ensues which mimics the effects of invasion and intercellular spread in the mucosa of the colonic epithelium (Mackel et al., 1961).

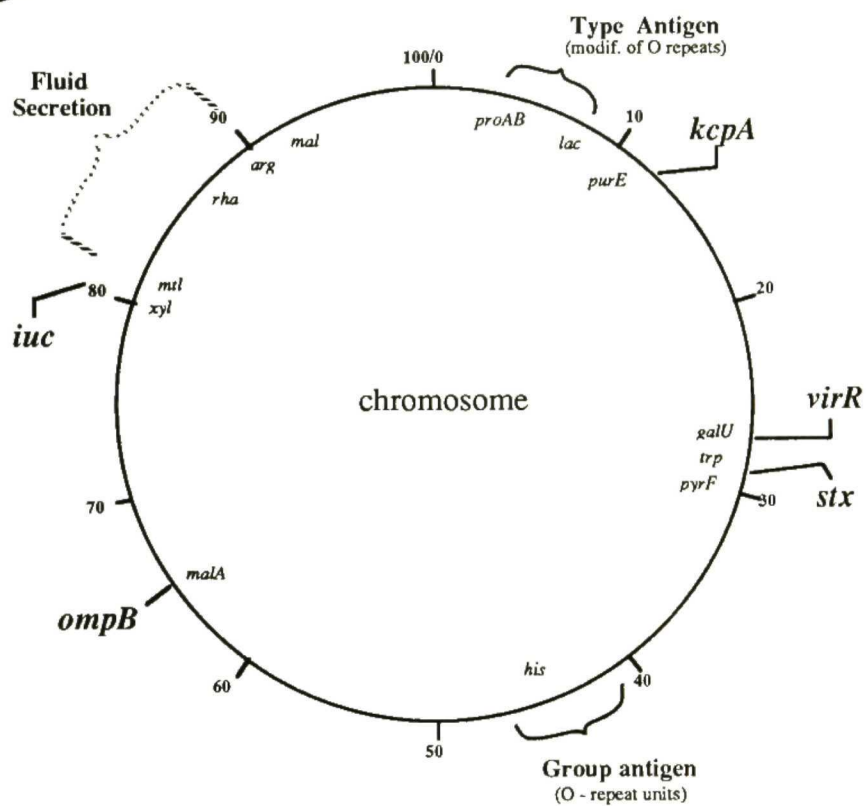
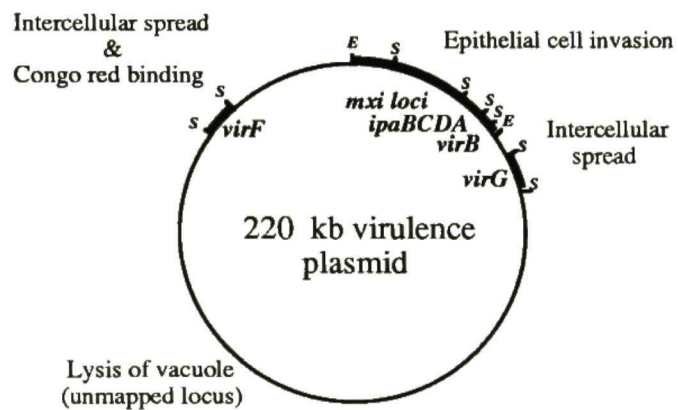
Genetics of *Shigella* virulence.

Studies which employed the assay systems mentioned above, along with classical genetic techniques and recombinant DNA technology, have revealed that *Shigella* species virulence is a multigenic phenomenon. Virulence loci and genes have been identified which are encoded both chromosomally and on a large 180 to 220 kilobase (kb) virulence plasmid (Fig. 2).

I. Chromosomal virulence loci

Early studies to determine the genetic basis of *Shigella* virulence took advantage of the close genetic relatedness of *Shigella* and *E. coli*. This extensive, though incomplete, genetic homology was first confirmed in studies which demonstrated conjugation between *E. coli* K-12 and *Shigella* strains (Luria and Burrous, 1957). Hybrid *Shigella* strains were isolated by conjugal transfer and integration of chromosomal DNA from non-pathogenic *E. coli* K-12 Hfr donor strains to virulent *S. flexneri* recipients. *Shigella* transconjugants were identified and screened for the alteration of any virulence phenotypes thereby identifying the map position of chromosomal loci which encoded virulence determinants. Reciprocal conjugations using *Shigella* Hfr strains as donors and *E. coli* K-12 strains as recipients were also performed (Schneider and Falkow, 1964). *E. coli* K-12 transconjugants were screened for the inheritance of virulence phenotypes which confirmed the location of virulence loci.

Figure 2. Organization of known genetic determinants of *Shigella* spp. Genetic loci (map position and genotype abbreviations) shown on the inside of the chromosomal map are those reported for *E. coli* K-12 (Bachmann, 1987). Chromosomal genetic loci shown in bold type are those identified for *Shigella* spp. and include: keratoconjunctivitis provocation (*kcpA*), virulence regulator (*virR*), Shiga toxin (*stx*), aerobactin (*iuc*), and the *ompB* locus. Virulence plasmid loci include: invasion plasmid antigens (*ipaBCDA*), membrane expression of Ipa (*mxi*), virulence gene activators (*virB* and *virF*), and *virG*. *E* and *S* represent the restriction sites for *Eco*RI and *Sal*I, respectively.



These experiments led to the identification of several chromosomal loci essential for the expression of a full virulence phenotype (Fig. 2). Subsequent characterization of these chromosomal regions have, in some cases, identified specific genes associated with virulence.

A. *xyl-rha* region

The *xyl-rha* region of the *Shigella* chromosome (80 to 88 min; Fig. 2) was the first chromosomal virulence locus to be identified. Hybrid *S. flexneri* 2a strains which incorporate the *E. coli* K-12 *xyl-rha* region are unable to persist within intestinal epithelial cells (Falkow *et al.*, 1963). Nevertheless, these hybrid strains are able to evoke a positive Séreny test and invade the intestinal mucosa which causes only a mild intestinal inflammation in monkeys fed this mutant strain (Formal *et al.*, 1965). Later studies demonstrated that in *S. flexneri*, *S. sonnei*, and *S. boydii*, expression of aerobactin and its 76 kilodalton (kDa) receptor protein are linked to the *mtl* locus at 81 minutes on the chromosome (Lawlor and Payne, 1984; Griffiths *et al.*, 1985). The expression of aerobactin, a high-affinity iron chelating hydroxamate compound or siderophore, has been implicated in the increased virulence of *E. coli* ColV strains (Williams, 1979). In *Shigella*, aerobactin operon (*iuc*) deletion mutants were initially shown to be avirulent in the chicken embryo model (Lawlor *et al.*, 1987), an assay system in which wild-type *Shigella* strains invade the embryo and produce a lethal infection when injected allantoically (Payne, 1989). Southern hybridization analysis of these deletion mutants revealed however, that sequences flanking the *iuc* operon were also deleted, which suggests that the loss of additional genes

may contribute to the avirulent phenotype observed in the *iuc*⁻ mutants (Lawlor *et al.*, 1987). Characterization of transposon-mediated *iuc* null mutants indicates that loss of aerobactin production does not alter the invasion phenotype of the mutant strains (Lawlor *et al.*, 1987; Nassif *et al.*, 1987). When compared to wild-type *Shigella*, *Iuc*⁻ transposon mutants require longer periods of time to reach the critical density necessary for invasion of the chick embryo (Lawlor *et al.*, 1987) and a larger inoculum to evoke keratoconjunctivitis (Nassif *et al.*, 1987). *iuc::Tn10* mutants are also impaired in their ability to cause fluid secretion and altered histopathology in ligated rabbit ileal loops (Nassif *et al.*, 1987), an assay routinely used to assess the effects of enterotoxins and the virulence potential of enteric pathogens. As in the Séreny test, higher concentrations of the mutant strain are required to exhibit a wild-type phenotype in the ligated ileal loop model. Based on these studies, it appears that the failure of shigellae to produce aerobactin affects the organism's virulence by altering the growth capacity of the strain within tissues in the extracellular compartment in an inoculum-dependent manner (Nassif *et al.*, 1987).

Other studies have shown that *E. coli* K-12 transconjugants which inherit a region overlapping the *xyl-rha* region of the *Shigella* chromosome, the *arg-mtl* (90 to 81 min.; Fig. 2), are able to cause fluid secretion in ligated rabbit ileal loops (Sansonetti *et al.*, 1982). These observations led to the conclusion that this region contained the Shiga toxin gene. However, later studies (see below), mapped the gene for Shiga toxin to 30 minutes on the *S. dysenteriae* chromosome (Sekizaki *et al.*,

1987). Therefore, the evidence provided by the *arg-mtl* transconjugants and the altered ability of *Iuc⁻* mutants to cause fluid secretion in the ligated ileal loop model implicate aerobactin as the most likely candidate for the virulence determinant mapping to this region of the chromosome. Nonetheless, a report of aerobactin deletion mutants being avirulent in the chicken embryo model (Lawlor *et al.*, 1987) suggests that genes involved in invasion remain unidentified within the *arg-mtl* region.

B. *lac-gal* region

The ability of virulent *S. flexneri* to evoke a positive Séreny reaction is associated with several virulence loci, one of which is located in a region between the *lac* (8 min.) and *gal* (17 min.) chromosomal markers, closely linked to *purE* (14 min.; Formal *et al.*, 1971). The virulence locus, identified by the construction of *S. flexneri* hybrids which incorporated the homologous *purE* region from *E. coli* K-12, was designated *kcpA* for keratoconjunctivitis provocation (Fig. 2; Formal *et al.*, 1971). As will be discussed later, the 220 kb virulence plasmid is required for HeLa cell invasion and loss of the virulence plasmid, despite the maintenance of the *kcpA* locus, renders the plasmidless strain non-invasive (Sansonetti *et al.*, 1982). *E. coli* K-12 hybrids which harbor only the virulence plasmid are HeLa cell invasive but remain localized in discrete areas of the HeLa cell cytoplasm and are, therefore, Séreny negative (Sansonetti *et al.*, 1983; Pál *et al.*, 1989). Introduction of the *S. flexneri kcpA* locus into these *E. coli* K-12 hybrids results in the strain's ability to invade and grow in a dispersed pattern in the HeLa cell cytoplasm (Pál *et al.*, 1989). Based on these results, it was concluded that expression of the *kcpA* locus in conjunction with the

expression of a plasmid-encoded virulence gene(s) facilitates the intracytoplasmic spread of invasive *Shigella* (Pál et al., 1989).

C. his

The genetic determinants for O-antigen biosynthesis in *E. coli* map to a chromosomal position closely linked to the *his* operon (Orskov and Orskov, 1962). In *S. flexneri*, O-antigen expression is linked to the *his* and *pro* chromosomal loci (Formal et al., 1970). In contrast, O-antigen biosynthetic genes of *S. sonnei* are encoded by the large Form I virulence plasmid (Kopecko et al., 1980), while in *S. dysenteriae* serotype 1, a 9 kb plasmid as well as chromosomal loci are required for somatic antigen biosynthesis (Watanabe and Timmis, 1984). The role for LPS O-side chain expression in *Shigella* virulence was assessed by mating virulent strains of *S. flexneri* serotype 2a with *E. coli* strains which express O8 or O25 somatic antigens (Gemski et al., 1972). Transconjugants which inherit the genes for the O8 somatic antigen are unable to evoke a positive Séreny reaction. In contrast, transconjugants which express the *E. coli* O25 somatic antigen remain fully virulent and are agglutinated by serotype 2a antisera. Based on the agglutination and virulence phenotypes of the *S. flexneri* O25 hybrid strain, the serotype 2a and O25 somatic antigens appear to be very similar in structure and function. The conclusion from these studies, therefore, was that expression of *S. flexneri* specific O-antigen was necessary for invasion of mammalian epithelial cells. Subsequent studies demonstrated, however, that rough strains (O-antigen negative), although unable to cause a positive Séreny test, were fully invasive for HeLa cells (Okamura and Nakaya, 1977). Thus, it was proposed that the presence of O-antigen, while not required for epithelial cell

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penetration, may play a role in bacterial virulence by conferring resistance to serum host defenses prior to invasion (Okamura and Nakaya, 1977).

D. *stx*

The gene coding for Shiga toxin, *stx*, has been mapped to approximately 30 minutes on the *S. dysenteriae* serotype 1 chromosome (Sekizaki *et al.*, 1987). Long recognized as one of the most potent bacterial toxins (O'Brien and Holmes, 1987), the definitive contribution of Shiga toxin to the clinical manifestations of shigellosis has only recently been determined (Fontaine *et al.*, 1988). These studies compared the virulence phenotypes of wild-type *S. dysenteriae* serotype 1 to toxin negative strains and demonstrated that expression of Shiga toxin has no effect on the ability of the wild-type strain to invade, multiply intracellularly, or kill HeLa cells. Significant pathological differences however, were observed in monkeys infected with the wild-type and toxin negative strains. Toxin expression is correlated with the presence of blood within stools and a sharp drop in blood polymorphonuclear cells. Histopathological alterations such as the destruction of capillary vessels within the connective tissue of the colonic mucosa and a major efflux of inflammatory cells to the intestinal lumen also strongly correlate with toxin expression. Thus, Shiga toxin significantly influences the severity of the *Shigella* infection by induction of colonic vascular damage (Fontaine *et al.*, 1988).

II. Extrachromosomal loci

A requirement for extrachromosomal virulence determinants in *Shigella* was first demonstrated when it was observed that virulent strains

of *S. sonnei* (Form I) contained a 180 kb plasmid that was absent in Form II derivatives which are always avirulent and devoid of LPS O-side chains (Sansonetti *et al.*, 1981). Mobilization of the large plasmid into rough, Form II *S. sonnei* results in transconjugants which are fully virulent (Sansonetti *et al.*, 1981) and which synthesize Form I LPS O-side chains (Kopecko *et al.*, 1981). Surveys of *S. flexneri*, *S. boydii*, *S. dysenteriae*, and EIEC strains indicate that virulent strains all harbor a large non-self transmissible plasmid (180 to 220 kb) which is mutated or absent in avirulent strains (Sansonetti *et al.*, 1982; Hale *et al.*, 1983; Sansonetti *et al.*, 1983). Although plasmids among these different strains exhibit a great deal of restriction endonuclease site heterogeneity, Southern hybridization analysis and plasmid mobilization studies indicate that the virulence plasmids of *Shigella* spp. and EIEC are highly homologous and functionally interchangeable (Hale *et al.*, 1983; Sansonetti *et al.*, 1983).

Conjugation of donor *Shigella* strains with *E. coli* K-12 recipients established that a full virulence phenotype can not be expressed by *E. coli* K-12 transconjugants in the absence of the *Shigella* virulence plasmid (Sansonetti *et al.*, 1983). Furthermore, *E. coli* K-12 transconjugants which harbor the *Shigella* virulence plasmid, in the absence of any *Shigella* chromosomal loci, are HeLa cell invasive (Sansonetti *et al.*, 1983). Therefore, the plasmid is necessary and sufficient for bacterial invasion of cultured mammalian cells.

Molecular characterization of the large plasmids has led to the identification of several genes required for the expression of plasmid-associated virulence phenotypes. In addition to invasion, the virulence

plasmid is required for escape from the endocytic vacuole, intracytoplasmic mobility, and intercellular spread. Contact hemolytic activity and binding Congo red dye are two plasmid-associated phenotypes which are also strongly correlated with virulence.

A. Invasion

Plasmid sequences necessary for invasion were first identified by employing a cosmid cloning strategy (Maurelli *et al.*, 1985). Recombinant plasmids carrying large fragments (30 to 40 kb) of the *S. flexneri* serotype 5 virulence plasmid were isolated and introduced into an avirulent, plasmidless strain of *Shigella* via a λ phage delivery system. Transductants carrying the recombinant plasmids were then screened for their ability to invade HeLa cells. Six independent recombinants which contain a common 37 kb sequence were isolated and shown to restore invasion of HeLa cells to plasmidless *Shigella* recipients. Based on these studies, the 37 kb core sequence was defined as the minimum sequence necessary for *Shigella* invasion (Maurelli *et al.*, 1985).

Two dimensional gel electrophoresis analysis of radiolabelled minicell protein extracts from virulent *S. flexneri* strains of serotypes 2a and 5, as well as a strain of EIEC serotype 0143, identified seven plasmid-encoded polypeptides designated a through g (Hale *et al.*, 1985). These same proteins are not detected in minicell preparations from plasmidless derivatives of the virulent strains. Four of these proteins a (M_r 78 kDa), b (M_r 62 kDa), c (M_r 43 kDa), and d (M_r 37 kDa) and a 140 kDa protein, were shown by Western blot analysis to be the primary immunogenic peptides recognized by convalescent sera from monkeys previously immunized with *S. flexneri* serotype 2a and children recovering from *S. flexneri* or

S. sonnei infections (Hale *et al.*, 1985; Oaks *et al.*, 1986). The cross-reactive nature of these convalescent antisera suggested that the immunogenic proteins are highly conserved in virulent strains of *Shigella* and EIEC. Evaluation of the serum immune response to *Shigella* proteins in Rhesus monkeys and humans revealed that convalescent sera exhibit high antibody titers against whole bacteria of *S. flexneri*, *S. dysenteriae* 1, *S. sonnei*, and EIEC (Oaks *et al.*, 1986). These observations substantiate previous conclusions that the immunogenic proteins are expressed by all virulent strains of *Shigella* and EIEC and suggest that at least some of the plasmid-encoded immunodominant proteins are expressed on the cell surface.

In an attempt to identify virulence plasmid genes which encode immunogenic polypeptides, a λ gt11 expression vector system was employed (Buysse *et al.*, 1987). Several recombinant phage were identified which expressed polypeptides b, c, and d. Characterization of the recombinant clones indicated that the cloned genes, designated *ipaB*, *C*, and *D* for invasion plasmid antigens, were closely linked and represented discrete transcription units (Buysse *et al.*, 1987).

Western blot analysis of transductants harboring one of the previously described cosmid clones, pHS4108, revealed that convalescent monkey sera recognizes the Ipa proteins (Maurelli *et al.*, 1985). Thus, the genes which encoded for these proteins were located within the 37 kb core invasion sequence. In later studies, Tn5 transposon mutants of pHS4108 were identified which exhibited reduced expression of IpaB, C, and D and were also reduced in their ability to invade HeLa cells (Baudry *et al.*, 1987). A mutant which no longer expressed IpaA, however, was

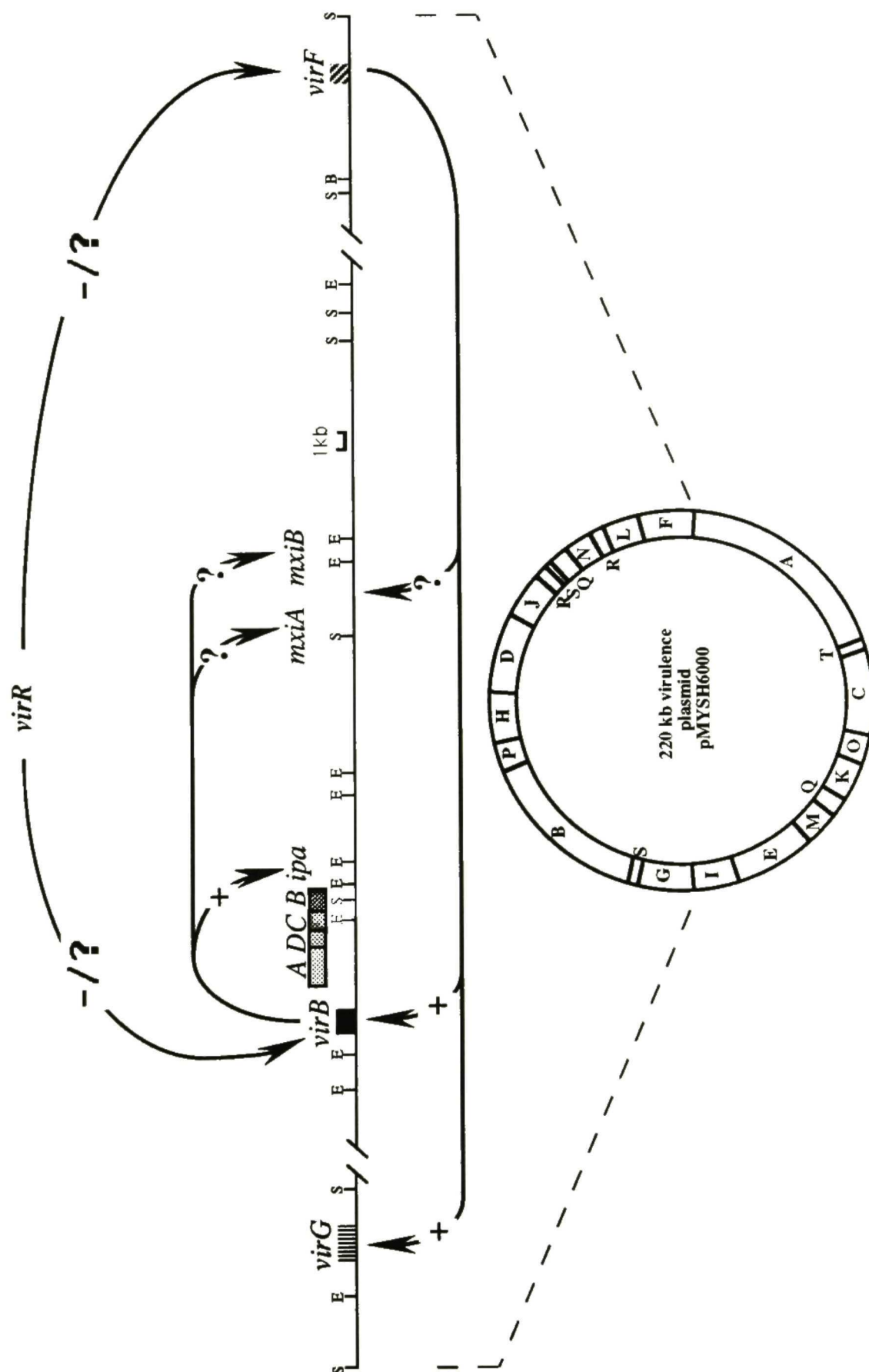
still invasive. From these observations, the expression of *ipaB*, *C*, and *D* was shown to be essential for the ability of *S. flexneri* to penetrate epithelial cells. In addition, the coding sequence of the *ipa* gene cluster was localized to the left hand portion of pHS4108 with the genes arranged in the order *ipaBCDA* (Fig. 3). Although the genetic maps of the *ipa* region from these studies and the previous study (Buysse *et al.*, 1987) were closely aligned in the position of restriction sites and in the gene order, contradictory results were obtained with respect to the transcriptional organization of the *ipa* gene cluster. One study proposed an operon model (Baudry *et al.*, 1987) and the other study proposed a model of independent transcriptional units (Buysse *et al.*, 1987).

Tn5 insertions which caused a loss of the invasive phenotype were also mapped to four other regions of pHS4108. In combination with the *ipa* coding region, these five distinct regions span approximately 20 kb and define specific plasmid virulence loci involved in epithelial cell invasion (Baudry *et al.*, 1987).

In addition to the identification of plasmid-specific virulence loci, a *SalI* restriction map of the *S. flexneri* serotype 2a virulence plasmid was constructed (Fig. 3; Sasakawa *et al.*, 1986a). Non-invasive Tn5 mutants of *S. flexneri* serotype 2a were isolated and mapped to four contiguous *SalI* fragments, B, P, H, and D (Sasakawa *et al.*, 1986b). Precise mapping of both non-invasive and invasive Tn5 insertions indicated that the non-invasive insertions could be separated into distinct virulence loci, designated Regions 1 through 5, which span approximately 31 kb of the virulence plasmid (Sasakawa *et al.*, 1988). These data, along with the restriction map of the plasmid sequence defined by Regions 1

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Figure 3. Temperature-dependent *Shigella* virulence gene regulatory network. (*Top*) chromosomal locus *virR*. (*Middle*) Portion of *S. flexneri* serotype 2a 220 kb virulence plasmid and designated virulence gene loci. Boxes denote coding sequences of each gene. (*Bottom*) *S. flexneri* serotype 2a virulence plasmid *SalI* restriction map. Arrows denote genes which are regulated by *virR*, *virB* and/or *virF*. Plus signs indicate positive regulation, minus signs indicate negative regulation, and question marks indicate that regulation is unknown.



through 5 (Sasakawa *et al.*, 1988), indicate that this region is homologous to the previously described cosmid clone pHS4108 (Baudry *et al.*, 1987). Independent studies of the functional organization and nucleotide sequence of the *ipa* genes or Region 2 confirmed previous results and also raised questions as to the role of *ipa* gene products in the invasive process (Baudry *et al.*, 1988; Venkatesan *et al.*, 1988; Sasakawa *et al.*, 1989). The deduced amino acid sequence of Ipa B, C, and D predicts proteins of 62 kDa, 41-43 kDa, and 37 kDa in size and is in agreement with previous reports (Hale *et al.*, 1985). Transcript mapping and nucleotide sequence analysis shows a complex transcriptional organization in this region with two additional open reading frames (ORF) being identified which code for proteins of 21-24 kDa and 18 kDa predicted molecular size. These data also suggest that *ipaB*, *C*, *D*, and *A* may be transcribed both individually and as a single transcriptional unit. Highly hydrophobic protein domains consistent with transmembranous location (Pugsley and Schwartz, 1985) were predicted from the *ipa* nucleotide sequences. However, no signal peptide sequences, as would be predicted for surface expressed or secreted proteins, were detected. These data suggest that the Ipa proteins may require additional protein products to facilitate their release to the outer portion of the organism.

Non-invasive transposon insertions in the *S. sonnei* Form I plasmid have also been identified which appear to be located in sites analogous to those described for pHS4108 (Watanabe and Nakamura, 1986). In addition, the restriction map of a 20 kb region defined by the transposon insertions exhibited a striking similarity between the Form I plasmid sequence and pHS4108. Other studies have confirmed that cloned sequences

from the Form I plasmid carried the *ipaBCDA* sequence (Kato *et al.*, 1989). Therefore, a 30 kb region of the *S. flexneri* serotype 5 virulence plasmid defined by pHS4108 appears to be conserved within the various *Shigella* spp.

B. Lysis of the phagocytic vacuole and intracellular multiplication

Electron micrographs of intestinal epithelial cells infected with *Shigella* reveal that prior to multiplying in the target cell cytoplasm, invading bacteria are first observed residing in cytoplasmic vacuoles (Takeuchi *et al.*, 1965). A wild-type *S. flexneri* serotype 5 strain and an *E. coli* K-12 strain which harbors the wild-type virulence plasmid (pWR100), multiply rapidly within infected HeLa cells, while BS169, a plasmidless derivative of *S. flexneri* serotype 5 harboring only pHS4108 (BS169/pHS4108), exhibits a slower rate of intracellular multiplication (Sansonetti *et al.*, 1986). Microscopic evaluation of HeLa cell monolayers infected with BS169/pHS4108 demonstrated that this strain requires a longer period of time to escape from the phagocytic vacuole when compared to both the wild-type and *E. coli* K-12/pWR100 strains. Thus, intracellular multiplication was shown to directly correlate with escape from the phagocytic vacuole and requires the presence of a complete virulence plasmid. In these and concurrent studies (Clerc *et al.*, 1986), epithelial cell invasion, rapid intracellular spread, and early and efficient lysis of the phagocytic vacuole by invasive strains of *S. flexneri*, *S. sonnei*, and *S. dysenteriae* were perfectly correlated with the degree to which these invasive strains were able to induce contact-mediated hemolytic activity. Based on the direct correlation of these three virulence phenotypes, it was concluded that plasmid-encoded contact-

mediated hemolytic activity provided the most likely mechanism for invasive shigellae to lyse the phagocytic vacuole (Sansonetti *et al.*, 1986)

C. Intracytoplasmic mobility and intercellular spread

Tn5 insertions which map to the *SalI* G restriction fragment of the *S. flexneri* virulence plasmid have also been identified. These *SalI*G::Tn5 mutants are still capable of epithelial cell invasion but unable to evoke guinea pig keratoconjunctivitis (Sasakawa *et al.*, 1986). Microscopic evaluation of these Tn5 insertion mutants indicates that their initial rate of intracellular replication is equivalent to that of a wild-type strain (Sasakawa *et al.*, 1986), yet they remain localized within the cytoplasm and eventually are cleared from the epithelial cells (Makino *et al.*, 1986). Fine mapping of random *SalI* G::Tn5 insertions and complementation analysis of mutations in this fragment identified a single gene designated *virG*. The *virG* gene product has been identified as a bacterial surface-exposed protein of M_r 130 kDa (Lett *et al.*, 1989). Nucleotide sequence analysis of a *virG* clone identified a single ORF large enough to generate a protein of 130 kDa molecular weight. Southern hybridization using an internal restriction fragment of the *virG* coding sequence as a probe, detected the presence of homologous sequences in all species of *Shigella* and strains of EIEC (Makino *et al.*, 1986) which further substantiated the idea that the virulence genes in these enteroinvasive pathogens are conserved. It is currently accepted that VirG is identical to the 140 kDa immunodominant protein (Oaks *et al.*, 1986). A plasmid locus designated *icsA* (intra cellular spread) has also been identified and characterized. Based on the size of the *icsA* protein

(120 kDa) and the phenotype of *icsA*⁻ mutants, it appears that *icsA* and *virG* are allelic (Bernardini *et al.*, 1989). More significantly, these studies demonstrated that invasive shigellae utilize host cell microfilaments to spread throughout the host cell cytoplasm and to contiguous cells in a HeLa cell monolayer. A phenotype similar to *VirG*⁻ mutants is observed in both infected HeLa cell monolayers treated with cytochalasin D (actin polymerization inhibitor) and in HeLa cell monolayers infected with *icsA*⁻ mutants. Intracellular bacteria also exhibit intense staining with N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phalloidin, a F-actin-specific dye, at their surface and trailing behind the bacteria as they move through the cell. Therefore, *VirG/IcsA* may function to anchor the bacteria to the microfilaments thereby facilitating bacterial spread within the host cell cytoplasm.

D. Congo red binding

The ability of shigellae to bind the dye Congo red is a plasmid-encoded phenotype used to distinguish virulent and avirulent strains. Virulent *Shigella* strains, capable of binding the dye incorporated into agar medium, appear as red colonies (*Pcr*⁺ or *Crb*⁺) while avirulent strains, incapable of binding the dye, appear as pink or white colonies (*Pcr*⁻ or *Crb*⁻; Payne and Finkelstein, 1977). Conversion from *Pcr*⁺ to *Pcr*⁻ is linked to either loss of, or deletions in, the 220 kb virulence plasmid and is accompanied by a loss of HeLa cell invasiveness (Maurelli *et al.*, 1984). Deletions in several different locations on the virulence plasmid cause a *Pcr*⁻ phenotype (Sasakawa *et al.*, 1986). Suppression of the *Pcr* defect by transduction of DNA sequences from a *Pcr*⁺ strain to *Pcr*⁻ mutants resulted in the isolation of *Pcr*⁺ transductants still unable to invade

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epithelial cells (Maurelli *et al.*, 1984). It appears, therefore, that a Pcr⁺ phenotype requires the presence of several virulence plasmid loci closely linked to genes required for invasion. The close linkage with invasion genes and the multigenic nature of Congo red binding is substantiated by the isolation of distinct Tn5 transposon mutants which are both Pcr⁻ and non-invasive (Sasakawa *et al.*, 1986) and the unsuccessful attempts to isolate the *pcr* structural gene(s) (Sakai *et al.*, 1986; Chambers *et al.*, 1985; Daskaleros and Payne, 1985). Virulence plasmid sequences have been isolated which confer a Pcr⁺ phenotype on Pcr⁻ strains of *E. coli* and complement Pcr⁻ mutations in *Shigella*. The Pcr⁺ *Shigella* transformants however, remain non-invasive.

Additional studies have demonstrated an association between Congo red binding and the ability of shigellae to bind hemin (Daskaleros and Payne, 1987). In these studies Pcr⁺ strains of *S. flexneri* serotype 2a were shown to bind hemin, while Pcr⁻ strains were incapable of doing so. Pre-binding of either hemin or Congo red to Pcr⁺ *S. flexneri* or the addition of hemin to HeLa cell monolayers enhanced the ability of these strains to invade HeLa cells in proportion to the amount of Congo red or hemin added. However, both Pcr⁺ and Pcr⁻ strains of *Shigella* can utilize hemin as a source of iron which suggests that Congo red binding and iron acquisition through hemin are not related. These observations further confound the precise role of dye binding in virulence.

Regulation of *Shigella* virulence genes.

Regulation of virulence gene expression in response to environmental signals has been described for several bacterial pathogens among which are

included the enteroinvasive *Shigella* species. Phenotypic alterations in the expression of *Shigella* virulence occur in response to changes in environmental growth conditions (Maurelli et al., 1984a). The molecular basis of these observed phenotypic changes has been studied extensively and has led to the formulation of an expanding network of regulatory genes which act to modulate the expression of a number of *Shigella* virulence genes.

I. Temperature

Temperature was first described as an environmental signal which effects the expression of *Shigella* virulence when it was demonstrated that shigellae, normally virulent when cultured at 37°C, become phenotypically avirulent when cultured at 30°C (Maurelli et al., 1984a). After being cultured at 30°C, virulent strains of *S. flexneri*, *S. sonnei*, and *S. dysenteriae* are neither able to invade epithelial cells nor to evoke keratoconjunctivitis in guinea pigs. Yet by shifting the growth temperature to 37°C, virulence is fully restored (Maurelli et al., 1984a). Re-expression of virulence was shown to require several generations of growth at 37°C and is dependent on *de novo* protein synthesis.

Operon fusion technology was used as a convenient means to determine the level at which regulation by temperature occurs. By a method similar to the one shown in Figure 4, random insertions of an operon fusion phage carrying a promoterless *lacZ* gene were generated in a virulent strain of *S. flexneri* serotype 2a (Maurelli and Curtiss, 1984). *lacZ* codes for the enzyme β -galactosidase and formation of positive operon fusions results in *lacZ* being placed under the transcriptional control of the target gene. β -galactosidase expression can then serve as an easy way to monitor and

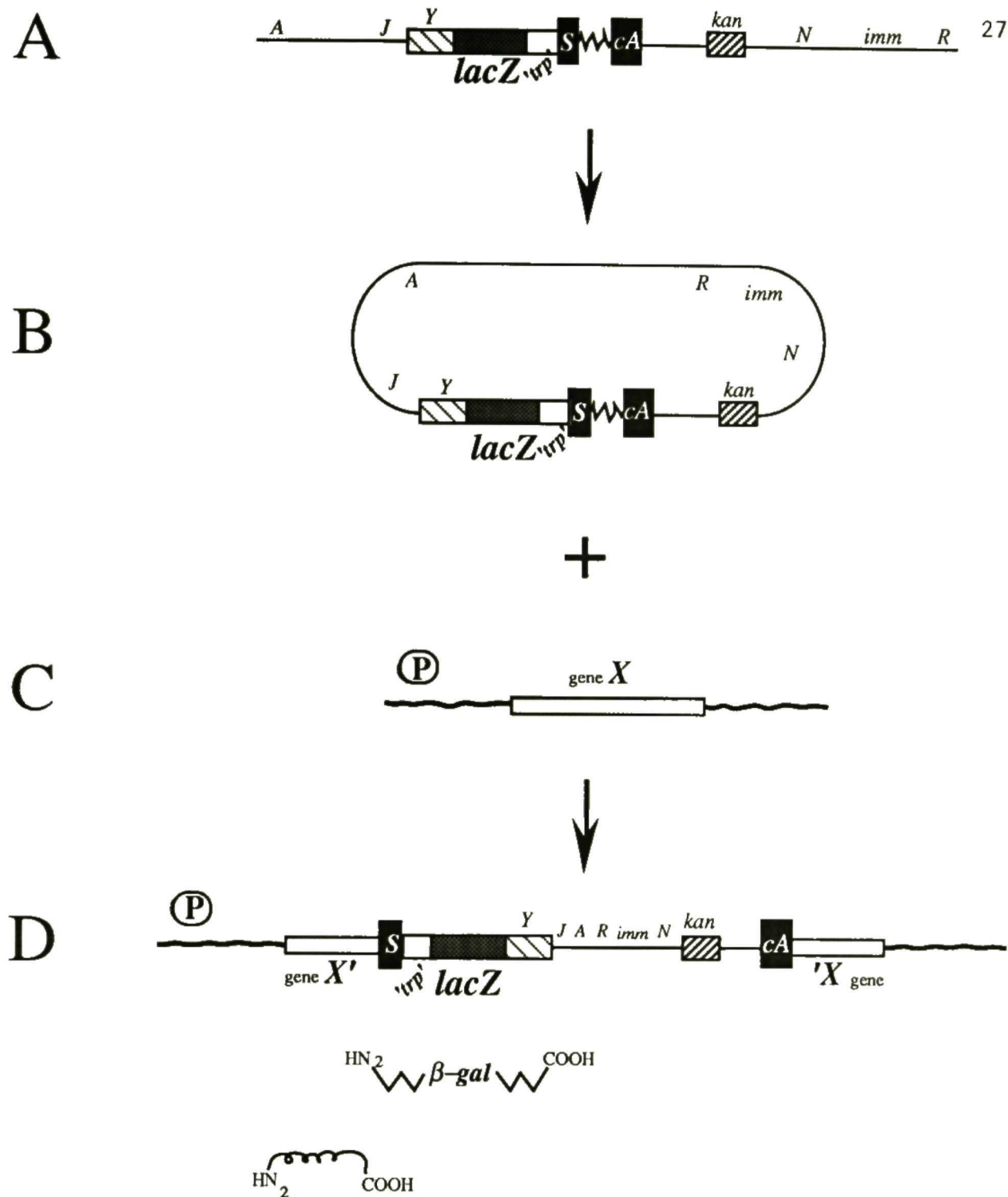


Figure 4. Formation of *lacZ* operon fusion. (A) *lacZ* operon fusion phage λ placMu53. Features include λ structural genes indicated by thin line; Mu phage sequences required for random insertion of phage genome into target gene (*S* and *cA*); promoterless *lacZ* encoding β -galactosidase; tryptophan operon ribosome binding site (*trp*) and the kanamycin resistance gene (*kan*). (B) Circularization of phage genome upon entry into bacterial cell. (C) Target gene (*X*) and target gene promoter (*P*). (D) insertion of phage genome into target gene and resulting *lacZ* operon fusion formation; truncated target gene protein and functional β -galactosidase.

quantitate the transcriptional activity of a target gene promoter. In this way a plasmid-encoded virulence gene (*vir*) was fused to *lacZ*. The formation of the *vir::lacZ* construct was directly linked to the loss of epithelial cell invasion and temperature-regulated β -galactosidase expression, which indicates that virulence gene regulation is at the transcriptional level.

A. *virR*

Random Tn10 mutagenesis of the *vir::lacZ* strain described above led to the identification of the chromosomally encoded regulatory locus, *virR* (Maurelli and Sansonetti, 1988). *virR::Tn10* mutants were identified as constitutively expressing β -galactosidase at 30°C. Transduction of the *virR::Tn10* mutation into a virulent strain of *S. flexneri* results in transductants which are deregulated for invasion of HeLa cells and are thereby able to invade, even after growth at 30°C. The *virR::Tn10* transductants also exhibit deregulated phenotypes in the plaque assay, Séreny test, and contact hemolytic activity. In addition, *ipaBCDA* expression is deregulated. Mapping of the *virR::Tn10* insertion placed the *virR* locus between *galU* and the *trp* operon at 27 min on the *S. flexneri* chromosome (Fig. 2). The strategy for cloning the *virR* locus exploited the close genetic linkage of *virR* and *galU*. A single cosmid clone was identified which complemented a *galU* mutation and was shown to restore temperature-regulated β -galactosidase expression in a *vir::lacZ* mutant harboring a *virR* deletion mutation. The results from these studies indicate that *virR* plays a central role in the temperature-dependent expression of *Shigella* virulence as a repressor of virulence gene expression at 30°C.

B. *virF*

virF was originally identified as a 1.0 kb region of the *S. flexneri* virulence plasmid, essential, but not sufficient to restore a Pcr⁺ phenotype in certain Pcr⁻ mutants of *Shigella* (Sakai *et al.*, 1986a). The *virF* gene was localized to the *SalI* F restriction fragment. Certain *SalI* F::Tn5 mutants were shown to be both non-invasive for HeLa cells and Séreny test negative, which indicates that *virF* is associated with virulence. Western blot analysis of the *SalI* F::Tn5 mutants demonstrated that *virF* mutants exhibit reduced levels of VirG and IpaB, C, and D and that wild-type levels of each of these proteins is restored by transformation of the mutant strain with a cloned *SalI* F fragment (Sakai *et al.*, 1988). *virF*, therefore, acts to positively regulate the expression of VirG and the IpaBCD proteins. Minicell analysis indicates that three proteins of *M_r* 30, 27, and 21 kDa are synthesized from the cloned *virF* and correspond in size to those predicted from the DNA sequence (Sakai *et al.*, 1986b). The 30 kDa VirF was later shown to be a transcriptional activator of *virG* expression (Sakai *et al.*, 1988). In the presence of cloned *virF*, *virG* mRNA levels and β -galactosidase expression from a *virG*::*lacZ* operon fusion are increased. The predicted amino acid sequence of VirF, however, does not exhibit significant protein sequence homology with any other known transcriptional regulator. Southern hybridization of virulence plasmid DNA from *S. sonnei*, *S. boydii*, *S. dysenteriae*, and EIEC strains, with a *virF* specific probe, revealed that *virF* homologous sequences could be detected in all of these bacterial species. These results, along with the identification of a gene in *S. sonnei* identical in function and DNA sequence to the *S. flexneri virF* (Kato *et al.*, 1989), suggest that the

mechanisms of virulence gene activation are conserved in *Shigella* and EIEC.

C. virB (ipaR/invE)

The *virB* locus was first identified by characterization of Tn5 transposon insertions within the *SalI* B restriction fragment of the *S. flexneri* serotype 2a virulence plasmid (Sasakawa *et al.*, 1986).

Specific *SalI* B::Tn5 mutants are unable to invade epithelial cells, evoke a positive Séreny test or bind Congo red (Adler *et al.*, 1989). The Tn5 insertions were mapped to the previously defined Region 1 (Sasakawa *et al.*, 1988). Cloned Region 1 sequences capable of restoring a wild-type virulence phenotype to the Region 1::Tn5 mutants were isolated and designated *virB* (Adler *et al.*, 1989). Minicell analysis of a *virB* clone revealed that a single protein of M_r 33 kDa was synthesized. Nucleotide sequence analysis of the *virB* recombinant identified a single ORF which corresponded to a protein of 35.4 kDa predicted molecular weight. Detailed characterization of Region 1::Tn5 mutants showed that in these strains expression of Ipa B and C is undetectable and expression of IpaD is reduced, whereas the expression of VirG is unaltered. Mutant strains transformed with cloned *virB* express wild-type levels of Ipa B, C, and D which correlates with the transformants' ability to invade epithelial cells. Region-1::Tn5 mutants also exhibit reduced levels of *ipaB*, *C* and *D* mRNA, which indicates that restoration of wild-type Ipa B, C, and D expression is due to the activation of *ipa* transcription conferred by the *virB* clone. Independent studies identified genes designated *ipaR* (Buysse *et al.*, 1990) from *S. flexneri* serotype 5 and *invE* from *S. sonnei* (Watanabe *et al.*, 1990) which are analogous in function and identical in

their predicted protein sequences to *virB*. Results from the *ipaR* study also demonstrated that *ipaA* expression is positively regulated by *ipaR*. Analysis of the predicted amino acid sequences of *ipaR* and *invE* reveal a striking amino acid sequence homology between these proteins and the bacteriophage P1 DNA binding protein, ParB. Thus, it appears that *virB* (*ipaR/invE*) serves as a transcriptional activator of certain plasmid-encoded virulence genes and may regulate these genes through a DNA binding mechanism. The initial characterization of *virB* also revealed that the level of *virB* transcripts in a *virF::Tn5* mutant are reduced and that introduction of a *virF* clone restores wild-type levels of *virB* mRNA (Adler *et al.*, 1989). On the basis of these observations, a dual transcriptional activator model for the regulation of virulence gene expression in *Shigella* was proposed (Fig.3). In this model, VirF alone is thought to positively regulate the expression of *virG* (Sakai *et al.*, 1989), while VirF activation of the *ipaBCD* genes occurs through the transcriptional activation of *virB* which directly activates expression of the *ipaBCD* genes (Adler *et al.*, 1989).

Recent unpublished studies have demonstrated that expression of both *virF* and *virB* are regulated by growth temperature (Yoshikawa *et al.* abstract presented at The 26th Joint Conference U. S.-Japan Cooperative Medical Science Program Cholera and Related Diarrheal Disease Panel, 1990) which suggests that the current model of plasmid virulence gene regulation may be expanded to include *virR*. In this model (Fig. 3) *virR* serves as the central regulator of the temperature-dependent response and regulates virulence gene expression via the two activators *virF* and *virB*.

D. Congo red

The exact role of Congo red binding in *Shigella* virulence is unknown. However, observations from a recent study suggest that Congo red binding may mimic the binding and activity of a host cell factor encountered by intracellular bacteria which serves to regulate expression of *Shigella* membrane proteins (Sankaran et al., 1989). Virulent *S. flexneri*, *S. dysenteriae* serotype 1, and EIEC strains, cultured in the presence of Congo red at 37°C, synthesize increased levels of membrane-associated proteins of M_r 43, 58, and 63 kDa. These proteins are not detected in bacteria cultured at 30°C even in the presence of Congo red, which suggests that the presence of the dye alone is insufficient to regulate expression of the membrane-associated proteins. Bacteria grown in the absence of Congo red, however, express the 43 kDa protein after invasion of tissue culture epithelial cells and guinea pig corneal epithelial cells. These studies suggest that a host cell intracellular component may cause a similar induction of protein expression in virulent strains of *Shigella* as the binding of Congo red dye but that this regulation is temperature dependent.

II. Osmolarity

Osmotic strength of the bacterial environment is one of the physical parameters that determines the ability of an organism to proliferate in a given habitat (Csonka, 1989). A recent study has demonstrated that virulence gene expression of *S. flexneri* is osmoregulated by the two-component transcriptional regulatory system OmpR-EnvZ (Bernardini et al., 1990). In *E. coli*, osmodependent transcriptional regulation of genes coding for the outer membrane porin proteins, OmpF and OmpC, requires the

positive regulatory activity of the OmpR and EnvZ proteins encoded by the *ompB* locus. OmpR and EnvZ represent a two-component regulatory system in which EnvZ is proposed to act as a membrane-associated osmosensor-protein kinase which phosphorylates the transcriptional activator OmpR thereby transducing the signal and facilitating the transcriptional activation of osmoregulated genes (Csonka, 1989). In *Shigella*, it has been shown that the transcriptional activity from a *vir::lacZ* operon fusion is osmodependent as β -galactosidase expression is induced under conditions of high osmolarity (Bernardini *et al.*, 1990).

The introduction of either an *ompB* deletion ($\Delta ompB$) or an *envZ::Tn10* mutation into an *S. flexneri* serotype 5 strain causes a dramatic reduction in the capacity of mutant strains to invade and multiply intracellularly while knocking out the ability to spread to contiguous cells in a HeLa cell monolayer or guinea pig cornea. The expression of the Ipa proteins in both of the mutant strains, however, remains unaltered. β -galactosidase expression is undetectable in either low or high osmolar conditions in a $\Delta ompB$ *vir::lacZ* strain. In contrast, in an *envZ::Tn10* *vir::lacZ* strain, β -galactosidase expression is reduced but still osmoregulated. A full virulence phenotype and osmoresponsive expression of β -galactosidase is restored by transformation of the mutant strains with an *ompB* clone. The results from this study suggest that both *ompR* and *envZ* are required for osmodependent regulation of certain *Shigella* virulence genes.

III. Intracellular environment

It is generally accepted that the environment encountered by invasive bacteria within a host cell is different from that outside of the

host cell. A recent report describing the effects of intracellular and extracellular growth on protein expression by *S. flexneri* (Headley and Payne, 1990), suggests that *S. flexneri* serotype 2a differentially expresses proteins when exposed to these two different environments. In these studies, radiolabelled bacteria isolated from infected HeLa cell monolayers exhibit increased expression of proteins of M_r 97, 62, 58, 50, 25, and 18 kDa while proteins of M_r 100, 85, 70, 64, and 55 kDa appear to be suppressed in their expression. The proteins suppressed during intracellular growth however, are fully expressed by bacteria grown in tissue culture media. Analysis of infected HeLa cell monolayers labelled at different times during the infectious cycle revealed that a bacterial protein of 58 kDa is induced during the invasion stage, while 62 and 25 kDa proteins are induced during intracellular multiplication. Radioimmunoprecipitation of bacterial proteins from intracellular and extracellular *S. flexneri*, with both convalescent monkey antisera and IpaB and IpaC specific monoclonal antibodies (MAbs), reveal that in the extracellular environment of tissue culture media all of the Ipa proteins are synthesized. In contrast, IpaB and IpaC expression is undetectable in organisms isolated from the intracellular environment while expression of an approximately 80 kDa protein corresponding in size to IpaA is induced. Additional proteins immunoprecipitated by the convalescent antisera include a protein of M_r 140 kDa corresponding to the size of VirG, a protein essential in the inter and intracellular movement of invasive shigellae. These studies suggest that IpaA and VirG may be induced during the organism's intracellular stage of pathogenesis where they may be required as virulence factors.

Purpose of these studies.

As presented in the previous sections, the current knowledge of *Shigella* virulence and its regulation is expanding rapidly. Certain questions remain unanswered. First, although a number of virulence genes have been identified, the precise number of plasmid and chromosomal genes involved in the pathogenicity of *Shigella* species is still unclear. Secondly, the number of these virulence genes which are temperature-regulated is undetermined. Thirdly, the precise chromosomal location and detailed characterization of the regulatory gene *virR* have yet to be determined. Finally, the questions of how bacteria detect changes in environmental temperature and how this information is translated into transcriptional regulation of virulence genes remain to be answered.

Therefore, the general purpose of these studies is to further elucidate the molecular mechanism by which *Shigella* spp. regulate virulence genes in response to growth temperature. To study temperature-regulated expression of *Shigella* virulence, the problem was approached from two directions: first, from the aspect of the target of regulation, the temperature-regulated virulence gene; and second, from the aspect of the central regulator, *virR*. Consequently, the presentation of these studies is divided into two parts as outlined below.

Part I: Identification and characterization of temperature-regulated virulence genes

A *lac* operon fusion system (Bremer *et al.*, 1985) was used to facilitate the identification and characterization of temperature-regulated virulence genes. Random insertions of the transposable

bacteriophage λ placMu53 (Fig. 4) in a virulent strain of *S. flexneri* 2a can be screened for positive *lacZ* operon fusions which i) cause a loss of virulence (due to insertional inactivation of the target gene) thereby identifying virulence genes, and ii) display temperature-dependent expression of β -galactosidase, indicative of operon fusions to temperature-regulated virulence gene promoters. The promoters identified by this system can subsequently be isolated by subcloning of the fusion end joint into a fusion promoter vector (Berman *et al.*, 1984). This would then permit studies to determine the molecular interaction, if any, of the target gene promoter and its regulatory proteins.

Four *virR*-regulated virulence genes essential for *Shigella* spp. invasion and three non-virulence associated genes were identified using the λ placMu operon fusion system (Hromockyj and Maurelli, 1989b). All four of the virulence genes identified mapped to the 220 kb *S. flexneri* serotype 2a virulence plasmid. One of the non-invasive fusions was precisely mapped to *ipaB*. The other three non-invasive mutants appeared to be altered in surface expression of the *ipa* virulence gene products and were thus designated *mxl* genes for membrane expression of *Ipa* (Hromockyj and Maurelli, 1989b). Subsequently, a temperature-regulated promoter from one of the *mxl::lacZ*⁺ fusion mutants was isolated and shown to require the presence of a virulence plasmid activator for its expression.

Part II: Characterization of *virR*.

virR has been identified as the central regulator of *Shigella* virulence gene expression in response to changes in temperature (Maurelli and Sansonetti, 1984). The second part of these studies focused on i) the

conservation of *virR* in a system other than *Shigella* in order to determine if a role exists for *virR* as a global regulator of non-virulence genes and, ii) the further characterization of *virR* in *Shigella* from the aspect of genetics and mechanism of action.

Heteroduplex analysis demonstrates that an overall DNA homology of approximately 90% exists between the *Shigella* and *E. coli* bacterial genera (Brenner *et al.*, 1972). Comparison of structural gene nucleotide sequence (Braun and Cole, 1982; Cossart *et al.*, 1986), electrophoretic analysis of enzyme polymorphisms (Ochman *et al.*, 1983), and serotyping and biotyping (Johnson *et al.*, 1975) all demonstrate the close genetic relationship between these two genera. In addition, EIEC are also temperature-regulated for virulence. A laboratory strain of *E. coli* K-12 therefore, was used to determine if a gene homologous in function to the *virR* gene of *S. flexneri* is conserved in non-pathogenic organisms. In these studies, non-pathogenic strains of *E. coli* were shown to carry a gene able to regulate the expression of a temperature-regulated *S. flexneri* virulence gene. Based on this evidence, it was concluded that a *virR* homolog existed in *E. coli* K-12 (Hromockyj and Maurelli, 1989a).

The *Shigella virR* locus was previously identified and cloned on a 30 kb chromosomal DNA fragment from a strain of *S. flexneri* serotype 2a (Maurelli and Sansonetti, 1988). In the present studies, further characterization of *virR* and its role in the regulation of *Shigella* virulence gene expression followed an approach similar to the one used previously to identify *virR* (Maurelli and Sansonetti, 1988). The *S. flexneri* reporter strain BS211 which carries a *vir::lacZ* operon fusion (Maurelli and Curtiss, 1984), together with a *virR* deletion, was used for

the identification of *virR* subclones derived from cosmid clone pATM003. β -galactosidase expression in BS211 is deregulated due to the *virR* deletion. *virR* subclones could therefore be identified by complementation of the *virR* defect and detected by the restoration of temperature-regulated expression of β -galactosidase. The smallest subclone which still retained *virR* activity could then be analyzed by nucleotide sequencing to identify the putative coding sequence. The derived nucleotide sequence of *virR* could then be analyzed and compared to other known regulatory proteins to potentially reveal the means by which *virR* responds to changes in temperature and then regulates virulence genes. The precise location of *virR* was identified using this strategy, and the gene responsible for the regulation of virulence was determined to be an allele of *hns*, a gene encoding a histone-like DNA binding protein (Pon *et al.*, 1988), induced by cold-shock (Jones *et al.*, 1987; VanBogelen *et al.*, 1990). Based on previous physio-chemical analysis of the H-NS protein and characteristics of *virR* clones determined in the studies presented here, a model for the mechanism of *virR* regulation is proposed. In addition, it was discovered that temperature-regulated virulence gene regulation could also be attained through multi-copy expression of the transfer RNA molecule for tyrosine (tRNA_{Tyr}).

MATERIALS AND METHODS

Bacterial strains and cultivation. The bacterial strains used are described in Table 2. All *Shigella* strains used in this study were derived from *S. flexneri* serotype 2a 2457T. Bacterial strains were routinely cultured in Luria Bertani broth (LB; 1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl; Davis *et al.*, 1980) or tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) at either 37°C or 30°C. Antibiotics were added to broth culture or agar media in the following concentrations: ampicillin, 30 to 100 µg/ml; kanamycin, 50 µg/ml; tetracycline, 5 µg/ml; chloramphenicol, 30 µg/ml. TSB agar medium was prepared by adding Bacto-agar (Difco) to TSB at a final concentration of 1.5%. Congo red (Sigma Chemical Co., St. Louis, MO) was added to TSB agar at 0.025% in order to determine bacterial dye binding. M9 salts (Davis *et al.*, 1980) was used as the base for preparing minimal media with carbon sources added to a final concentration of 0.5%. For growth of *Shigella*, minimal medium was supplemented with 10 µg nicotinic acid per ml (Sigma). Screening of *virR* subclones in strain BS211 (Table 2) was routinely performed on Bacto-MacConkey lactose agar (Difco).

Enzymes and radionuclides. All restriction enzymes and DNA modification enzymes used in these studies, unless otherwise indicated, were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Radionuclides ³²P and ³⁵S, used for Southern blot hybridization and dideoxy nucleotide sequencing respectively, were purchased from Amersham Corp. (Arlington Heights, Ill.).

TABLE 2. Bacterial strains.

Strains	Relevant genotype or description ^a	Reference or source
<i>E. coli</i> K-12		
DH5 α	$\phi 80d \Delta(lacZYA-argF) hsdR17 (r_k^- m_k^-)$	BRL ^b
C600	F ⁻ <i>thi-1 thr-1 leuB6 lacY1 tonA53 supE44</i> λ^-	Maniatis <i>et al.</i> , 1982
MG1655	F ⁻ λ^-	Bachmann, 1987
ATM016	F ⁻ <i>lacY1 glnV tyrT</i> $\Delta galU trp srl recA56 metB1$	Maurelli and Sansonetti, 1988
NF3079	<i>dam-3 araD13S9</i> $\Delta(araABOIC-leu)$ 7679 <i>rpsL</i>	Rowley and Wolf, 1991
	<i>galU galK</i> $\Delta(lac)$ X74	
<i>S. flexneri</i> 2a		
2457T	Wild-type harboring 220 kb virulence plasmid	Formal <i>et al.</i> , 1958
BS103	pSf2a140	Maurelli and Curtiss, 1984
BS181	2457T cured of pSf2a140	Maurelli and Sansonetti, 1988
BS184	2457T <i>Mal</i> ⁺ λ^S	Maurelli and Curtiss, 1984
BS185	2457T pSf2a140::Mud83 <i>Mal</i> ⁺ λ^S	Maurelli and Sansonetti, 1988
BS189	BS184 <i>virRI::Tn10</i>	Maurelli and Sansonetti, 1988
BS201	2457T <i>virRI::Tn10</i>	This study
BS211	2457T <i>recA56</i>	This study
	BS184 $\Delta virRI$	

TABLE 2. continued.

Strains	Relevant genotype or description ^a	Reference or source
<i>S. flexneri</i> 2a		
BS214	2457T Δ (<i>virRI-trp</i>)	Hromockyj and Maurelli, 1988a
BS226	2457T pSf2a140 Φ (<i>mxi::lacZ</i> ⁺ 11.5)	Hromockyj and Maurelli, 1988b
BS228	2457T pSf2a140 Φ (<i>mxi::lacZ</i> ⁺ 17.6)	Hromockyj and Maurelli, 1988b
BS230	2457T pSf2a140 Φ (<i>mxi::lacZ</i> ⁺ 17.7)	Hromockyj and Maurelli, 1988b
BS232	2457T pSf2a140 Φ (<i>mxi::lacZ</i> ⁺ 18.15)	Hromockyj and Maurelli, 1988b
BS234	2457T pSf2a140 Φ (<i>lacZ</i> ⁺ 17.9)	Hromockyj and Maurelli, 1988b
BS236	2457T pSf2a140 Φ (<i>lacZ</i> ⁺ 18.11)	Hromockyj and Maurelli, 1988b
BS238	2457T pSf2a140 Φ (<i>lacZ</i> ⁺ 18.13)	Hromockyj and Maurelli, 1988b
BS255	2457T pSf2a140::Mud83 Mal ⁺ λ^S Δ <i>virRI recA56</i>	Maurelli and Sansonetti, 1988
BS303	BS226 <i>virRI::Tn10</i>	Hromockyj and Maurelli, 1988b

^a Φ indicates presence of an operon fusion to the gene indicated; all operon fusions shown contain an insert of the fusion bacteriophage λ placMu53.

^b BRL, Bethesda Research Laboratories

Bacterial transformation. (i) *S. flexneri*: Transformation of *S. flexneri* strains was performed by the method described by Dagert and Erlich (Dagert and Erlich, 1979). The *S. flexneri* strain to be transformed was subcultured from a fresh overnight culture (37°C with aeration) at a 1:100 dilution into fresh LB or TSB and grown at 37°C with aeration to a final concentration of 8×10^8 to 9×10^8 bacteria per ml ($\text{O.D.}_{600\text{nm}} = 0.8$ to 0.9). Cultures were heated to 50°C for 10 minutes to inhibit bacterial restriction and enhance transformation efficiency before being placed on ice for 30 to 120 minutes. Bacteria were pelleted by centrifugation at $2,200 \times g$ for 5 minutes at 4°C. Supernatants were decanted and bacterial pellets were suspended by gentle vortexing in one half the original culture volume in an ice-cold, sterile solution of 100 mM MgCl_2 . Centrifugation was repeated and bacterial pellets were suspended in one half of the original culture volume in an ice-cold, sterile solution of 100 mM CaCl_2 - 50 mM MgCl_2 , followed by incubation on ice for 10 minutes. Bacteria were pelleted and suspended in 1/20 original culture volume in an ice-cold, sterile solution of 100 mM CaCl_2 and stored overnight (12 to 22 hours) before transformation. Competent bacteria were transformed by adding 100 to 500 ng of DNA, in a volume no greater than 10 μl , to 300 μl (approximately 3.0×10^9 bacteria) of competent bacteria and the mixture was chilled on ice for 30 minutes followed by a heat shock at 42°C for 5 minutes. Transformed bacteria were then chilled on ice for 2 minutes before being diluted 10-fold with LB, prewarmed to 37°C. Samples were then incubated at 37°C without aeration for 60 minutes to allow for expression of antibiotic resistance. Following this incubation step, bacteria were pelleted by centrifugation at $2,800 \times g$ for 1 minute at 4°C

and suspended to a final concentration of approximately 3.0×10^9 bacteria per ml and plated on appropriate selective media.

(ii) *E. coli* strain DH5 α : Transformation of *E. coli* strain DH5 α followed the method described by Hanahan (Hanahan, 1985). Fresh overnight broth cultures of DH5 α were subcultured in LB supplemented with 10 mM MgCl₂ - 10 mM MgSO₄ and grown at 37°C with aeration. Bacterial cultures were grown to a density of 4×10^7 to 7×10^7 bacteria per ml (O.D._{550nm} = 0.45 to 0.5) and immediately placed on ice for 10 minutes. Cultures were centrifuged at $2,200 \times g$ for 5 minutes at 4°C, supernatants were decanted, and the bacterial pellets were suspended to 1/3 of the original culture volume in ice-cold, filter sterilized, Standard Freezing Buffer (SFB; 10 mM CH₃COOK [pH 7.0], 100 mM RbCl, 45 mM MgCl₂·H₂O, 10 mM CaCl₂·H₂O, 3 mM [Co(NH₃)₆]Cl₃, 10% glycerol; pH was adjusted to 6.4 with 0.1 N HCl). Bacteria were incubated on ice for 15 minutes, centrifuged as before, and the bacterial pellets were suspended to 1/15 of the original culture volume in ice-cold SFB. Fresh dimethyl sulfoxide (DMSO; Sigma) was then added to a final concentration of 4.0% and bacteria were incubated on ice for 5 minutes. A second equal volume of DMSO was added and the bacteria were again incubated on ice for 5 minutes. Small volumes of the bacterial preparation were then flash frozen in a dry ice-ethanol bath in Falcon 2063 or 2059 tubes (Becton Dickinson and Company, Lincoln Park, NJ) and stored at -70°C until needed. Transformations were performed by thawing frozen competent bacteria on ice followed by the addition of DNA (10 pg to 100 ng/ 200 μ l of bacteria). The transformation mixtures were incubated on ice for 30 minutes followed by a 1 to 2 minute heat shock at 42°C. The bacteria were then chilled on ice for 2 minutes before being

diluted 10-fold with SOB media (2% [wt/vol] Bacto tryptone, 0.5% [wt/vol] yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose) prewarmed to 37°C and incubated with gentle aeration at 37°C for 1 hour to allow for the expression of antibiotic resistance. Transformed bacteria were then plated on appropriate selective media.

(iii) Other *E. coli* strains: *E. coli* strains other than DH5 α were transformed by the previously described calcium chloride procedure (Maniatis et al., 1982). Briefly, overnight cultures of the *E. coli* strain to be transformed were grown in liquid culture at 37°C with aeration and then diluted 1:100 in fresh media and grown under the same conditions the following day. The bacterial cultures were grown to a concentration of approximately 5×10^7 to 6×10^7 bacteria per ml (O.D._{550nm} = 0.45 to 0.5) and immediately chilled on ice for 10 minutes. The bacterial suspensions were pelleted by centrifugation at 4,000 x g for 5 minutes at 4°C and the supernatants were discarded. The bacterial pellets were suspended in half of the original culture volume of an ice-cold sterile solution of 50 mM CaCl₂ - 10 mM Tris hydrochloride (pH 8.0) followed by incubation on ice for 15 minutes. The bacterial suspensions were pelleted as before and bacterial pellets were suspended in 1/15 the original culture volume of an ice-cold, sterile solution of 50 mM CaCl₂ - 10 mM Tris hydrochloride (pH 8.0) followed by incubation on ice for 12 to 24 hrs. Competent bacteria were transformed by adding 100 to 500 ng of DNA, in a volume no greater than 10 μ l, to 200 μ l (2.8×10^9 to 3.2×10^9 bacteria) competent bacteria. The mixture was chilled on ice for 30 minutes followed by heating to 42°C for 5 minutes. Transformed bacteria were then chilled on ice for an additional 2 minutes before being diluted

10-fold with LB, prewarmed to 37°C. Transformation samples were then incubated at 37°C without aeration for 60 minutes to allow for expression of antibiotic resistance. Following this incubation step, bacteria were pelleted by centrifugation at 2,800 x g for 1 minute at 4°C and suspended to a final concentration of approximately 3.0×10^9 bacteria per ml and plated on appropriate selective media.

Virulence Assays. (i) Invasion: The invasion phenotype of *S. flexneri* was measured by the ability of bacteria to invade tissue culture monolayers of HeLa cells. The HeLa cell line used in these studies was kindly provided by T.L. Hale (Walter Reed Army Institute of Research, Washington, D.C.). The assay protocol was slightly modified from the one previously described (Hale and Formal, 1981). HeLa cells were maintained in basal Eagle medium (MEM; Whitaker M.A. Bioproducts Inc., Walkersville, MD) supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin at 37°C under 5% CO₂. Cells were plated at 4×10^5 cells per tissue culture dish (35 mm x 10 mm; Corning, Cambridge, MA) in supplemented MEM without antibiotics and grown at 37°C under 5% CO₂ for 18 hours before infection with bacteria. Bacteria for the invasion assay were cultured in TSB overnight at 30°C or 37°C and then were subcultured at a 100-fold dilution and grown at the same temperatures for approximately four generations. Bacteria were washed once in phosphate buffered saline (PBS; Whitaker M.A. Bioproducts Inc.), and suspended in 1.5 ml (3×10^8 bacteria) of prewarmed MEM. Prior to the addition of bacterial suspensions, the HeLa cell monolayers were washed twice by the addition of MEM prewarmed to 37°C, followed by gentle

agitation of the dishes. After aspiration of the final wash, bacterial samples (1.5 ml) were added to the HeLa cell monolayers in the tissue culture dishes. Dishes were immediately centrifuged at room temperature for 10 minutes at 3,000 rpm ($2,200 \times g$) in a Sorvall RT6000B centrifuge (DuPont, Wilmington, DE). The dishes were then incubated for 2 hours at 37°C without CO_2 . Following the 2 hour incubation period, HeLa cell monolayers were washed 5 times with PBS, fixed with methanol (5 minutes at room temperature), and stained with Giemsa (Sigma) solution. Fixed and stained HeLa cells were examined by light microscopy for the presence of intracellular bacteria. Invasion of $>80\%$ of the HeLa cell monolayer challenged by bacteria was considered positive for invasion.

(ii) Plaque assay: Plaque formation in HeLa cell monolayers was measured by the method previously developed (Oaks *et al.*, 1985). HeLa cells were maintained as described for the invasion assay. Culture dishes (60 mm x 10 mm dishes; Corning) however, were seeded at a concentration of 1.3×10^6 HeLa cells per dish and incubated at 37°C under $5\% \text{CO}_2$ for 48 hours. This allowed for the formation of a confluent monolayer of HeLa cells by the time of the assay. Overnight broth cultures of the test strains were grown at 30 or 37°C with aeration and subcultured at a 1:100 dilution in TSB and grown at the same temperature the following day. Bacteria were harvested when a final bacterial concentration of 1×10^6 to 3×10^6 bacteria per ml was attained. All test samples were adjusted to the same concentration (approximately 3×10^8 bacteria per ml; $\text{O.D.}_{600\text{nm}} = 0.3$) and washed twice in PBS. The bacterial pellets were suspended in MEM by vortexing and each sample was titered by plating on appropriate selective media. Prior to the addition of bacterial suspensions, the HeLa

cell monolayers were washed twice by the addition of MEM prewarmed to 37°C, followed by gentle agitation of the dishes. After aspiration of the final wash, bacteria were added to duplicate HeLa cell monolayers at approximate final concentrations of 3×10^5 and 3×10^4 bacteria per dish in 1.5 ml MEM. Dishes were rocked back and forth to evenly distribute the bacteria and incubated at 37°C at 5% CO₂ for 90 minutes. Following the 90 minute incubation, an agarose overlay was added (0.5% agarose, 5.0% fetal calf serum, 20 µg/ml gentamicin, in MEM with 0.45% glucose). The agarose was allowed to solidify for 10 minutes at room temperature before placing dishes at 37°C under 5% CO₂. Plaques were clearly visible at 48 hours at which time they were counted and plaque size and morphology were noted. Plaquing efficiency was expressed as the number of total plaques per input colony forming units.

(iii) Séreny test: This test measures the ability of invasive strains of bacteria to cause keratoconjunctivitis in guinea pigs (Séreny, 1955). Briefly, bacterial test strains were streaked for confluency on TSB agar from fresh overnight cultures grown in TSB at 37°C with aeration and incubated at 37°C overnight. Sterile cotton tipped swabs were used to remove a sample of the bacterial lawns to inoculate the right eye of each guinea pig (Hartley male or female ≈250 gm.; Hazelton Biologics Inc., Lenexa, KS). Left eyes served as uninoculated controls. Inoculated guinea pigs were kept for observation in Horsfal incubators (Hartford Metal Co., Aberdeene, MD). Guinea pigs were monitored for the production of keratoconjunctivitis (redness of eye, swelling, purulent exudate, and eye closure) for 5 days. All samples were assayed in triplicate. A positive Séreny test was the production of keratoconjunctivitis within 24

hours after bacterial infection.

(iv) Assay for Contact Hemolytic Activity. Contact hemolytic activity was determined by the method previously described (Clerc *et al.*, 1986). Fresh overnight cultures grown at 37°C with aeration were subcultured 100-fold into 10 ml of TSB and grown to late exponential phase ($\text{O.D.}_{600\text{nm}} = 1.0$; 1×10^9 bacteria per ml). Bacterial cultures were pelleted by centrifugation at $2,200 \times g$ for 5 minutes at 25°C and washed twice in PBS. The bacterial pellets were suspended in PBS to a final concentration of 1×10^{10} to 2×10^{10} bacteria per ml. Sheep red blood cells (SRBC) were prepared by washing pelleted cells (centrifuged at 2,000 rpm for 5 minutes at room temperature) twice in PBS and adjusting the final concentration to 4×10^9 SRBC per ml in PBS. Bacterial suspensions, in a volume of 50 μl , were dispensed into wells of a 96 well flat-bottom, polystyrene, microtiter plate (Costar) followed by the addition of an equal volume of SRBC. Control wells contained SRBC and PBS alone. Microtiter plates were vigorously agitated for 5 minutes at room temperature on a Mini-orbital Shaker (Bellco Glass Inc., Vineland, NJ). The microtiter plates were then centrifuged at $2,200 \times g$ for 10 minutes at 4°C, to bring bacteria into close contact with the SRBC before incubating the plates for 2 hrs at 37°C without shaking. Bacteria-SRBC pellets were then loosened by shaking as before followed by the addition of 150 μl of cold PBS. Samples were centrifuged at $2,200 \times g$ for 15 minutes at 4°C and 100 to 150 μl of each sample supernatant was transferred to a clean microtiter plate. Hemolytic activity was measured by obtaining the optical density of each supernatant at 540 nm on a Titertek Multiscan MK spectrophotometer (Flow Laboratories, Costa Mesa, CA).

Phage lysates and generalized transduction. Preparation of Plvir lysates and generalized transduction with Plvir followed the methods described previously (Silhavy *et.al.*, 1984). Donor strains were grown overnight in LB at 37°C with aeration. The following day strains were subcultured by diluting 100 fold in LB containing 5 mM CaCl₂ and cultured under the same conditions to a final concentration of 2×10^8 bacteria per ml. An existing Pl lysate was then used to infect 2×10^8 bacteria (1.0 ml) at a multiplicity of infection (MOI) of 50. Phage were allowed to adsorb to the bacteria for 20 minutes at 37°C without shaking. A volume of 200 μ l of the infected cells was transferred to a tube containing 2.5 ml pre-warmed (45°C) L-soft agar (1% tryptone, 0.5% NaCl, 0.5% yeast extract, 0.1% glucose, 5 mM CaCl₂, and 0.6% agar, [pH 7.0]) and poured onto L-agar media (1.2% agar) containing 5 mM CaCl₂, tilting the plate rapidly in order to spread the soft agar evenly. The soft agar was allowed to solidify for 5 minutes at room temperature before the plates were incubated at 37°C. The phage lysates were harvested after 6 hours or when the bacterial lawns cleared. The soft-agar overlays were scraped into a sterile centrifuge tube followed by two washes of the agar surface with 1.0 ml of a sterile solution of 10 mM MgSO₄. Any remaining bacteria in the harvested lysates were lysed by the addition of 0.5 ml chloroform followed by 1 minute of vigorous vortexing. Lysates were left at room temperature for 5 minutes and cleared of bacterial debris and agar by centrifugation at 12,000 x g for 10 minutes at 4°C. Cleared phage lysates were decanted into sterile tubes and stored at 4°C over a few drops of chloroform.

Recipient strains were grown under the same conditions as the donor strains. A volume of 1.5 ml of an overnight bacterial culture (1×10^9 to

2×10^9 bacteria per ml) was centrifuged ($2,800 \times g$ for 1 minute at 4°C) and the bacterial pellet was suspended in 0.75 ml of a sterile solution of 10 mM MgSO_4 - 5 mM CaCl_2 . A volume of 0.1 ml of the suspended bacteria was then mixed with transducing phage at an MOI of 1 to 3 and the phage were allowed to adsorb to the bacteria at 37°C for 20 minutes without shaking. To prevent reinfection of the bacteria, 100 μl of 1 M sodium citrate was added after adsorption. Bacteria were pelleted as previously and supernatants were aspirated. Pellets were suspended in 0.1 ml LB containing 20 mM sodium citrate before plating on the appropriate selection media. Transductions requiring expression of antibiotic resistance followed a modified procedure in which the transduced bacteria were suspended in 1.0 ml of LB containing 10 mM sodium citrate. The bacterial suspensions were then incubated 60 to 90 minutes at 37°C without aeration followed by plating of the transductants as described above.

Western blot. Whole bacterial extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane filters (Bio-Rad, Richmond, CA) as described previously (Burnette, 1981). Whole bacterial lysates were prepared from overnight cultures grown in TSB at 37°C . A volume of 10 ml of the overnight cultures were washed once in PBS and standardized to a final $\text{O.D.}_{750\text{nm}} = 0.8$. Bacteria were pelleted by centrifugation at $10,000 \times g$ for 2 minutes at 4°C and lysed by the addition of hot (100°C) sample buffer (phosphate buffer [50 mM K_2HPO_4 :50 mM Na_2HPO_4 70:30; pH 7.2], 2% SDS, 12% glycerol, 0.01% bromophenol blue, 5% β -mercaptoethanol). The lysed bacteria were then heated at 100°C for 4 minutes to ensure complete bacterial lysis.

Equal volumes of each sample were subjected to SDS-PAGE in 14% polyacrylamide slab gels.

Nitrocellulose filters were prepared for treatment with primary antibody as previously described (Mills *et al.*, 1988). The primary antibody used was either convalescent serum from a patient who had been infected with *S. flexneri* 2a or monoclonal antibodies (MAbs) specific for either IpaB or IpaC. Proteins were transferred by electroblot to nitrocellulose membranes for 90 minutes at 0.5 A. Following protein transfer, the membranes were incubated in casein "filler" (2% casein, 30 mM NaOH and 0.7 X PBS) with gentle shaking for 15 to 30 minutes at room temperature to block any non-specific antibody binding. Filter membranes were then immersed in convalescent antiserum diluted 100-fold in casein "filler" as the primary antibody in order to detect expression of the four major antigenic polypeptides encoded by the virulence plasmid pSf2a140 of 2457T. MAbs 2F1, 1H4, 4C8 (IpaB-specific), and 5H1 (IpaC-specific; Mills *et al.*, 1988) were used to specifically identify two of the four major antigenic polypeptides produced by *Shigella*. The MAbs in mouse ascites fluid were used at a 500-fold dilution in casein "filler". Filters were exposed to the primary antibody overnight at room temperature with gentle shaking. Following exposure to the primary antibody, filters were washed twice in PBS for 5 to 10 minutes at room temperature with shaking. Filters were then incubated with gentle shaking in alkaline phosphatase-conjugated staphylococcal protein A (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1000-fold in casein "filler" for 1 hour at room temperature. The membranes were again washed twice in PBS followed by a single wash in 50 mM Tris hydrochloride (pH 8.0) then exposed to the

phosphatase substrate (Naphthol AS-MX phosphate, 1 mg/ml, and Fast red TR salt, 2 mg/ml; Sigma) as described (Mills *et al.*, 1988). The enzymatic reaction was then stopped by rinsing the developed blot with tap water.

ELISA. The whole cell ELISA was performed following the procedure previously described (Mills *et al.*, 1988). Bacterial strains to be tested were cultured overnight in TSB at 37°C with aeration. Cultures were pelleted by centrifugation at 4,000 x *g* for 5 minutes at room temperature and the bacterial pellets were suspended in PBS to a final O.D._{600nm} = 5.0. Bacterial samples were added in a volume of 100 µl to wells of a 96 well flat bottom polystyrene microtiter plate (Corning) and incubated at room temperature for 1 to 2 hours to allow for bacterial adherence to the plastic surface. The wells were then emptied and an equal volume (100 µl) of casein "filler" was added to each sample well in order to block any non-specific antibody binding. Plates were incubated for 30 to 60 minutes at room temperature. Following incubation, the sample wells were then emptied and washed twice with PBS containing 0.05% Tween. MAbs 2F1 or 2G2, diluted 250-fold in casein "filler", were added to the sample wells in a 50 µl volume and the plates incubated at 37°C for 2 hours. The primary antibody was removed by emptying the wells and washing twice with PBS containing 0.05% Tween. An equal volume (50 µl) of goat anti-mouse IgG-alkaline phosphatase conjugate (Boehringer Mannheim) diluted 100-fold in casein "filler" was then added to each sample as the secondary antibody at room temperature for 2 hours. Samples were again washed before the addition of 50 µl of a solution of the chromogenic alkaline phosphatase substrate, p-nitrophenyl phosphate (Sigma), at a concentration of 1 mg/ml

in diethanolamine buffer (8.5% diethanolamine, 0.02% sodium azide, 0.01% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$). Samples were incubated at room temperature for 30 minutes before the colorimetric reaction was assessed photometrically at 405 nm in a Titertek Multiscan MK spectrophotometer (Flow Laboratories). Bacterial samples were tested in quadruplicate.

Mutagenesis by $\lambda\text{placMu53}$ and screening for *inv::lacZ* operon fusions. Random insertions of the transposable phage $\lambda\text{placMu53}$ were isolated in BS181 as previously described (Bremer *et al.*, 1985). An overnight culture of strain BS181 (1×10^9 to 2×10^9 bacteria) grown in LB containing 0.2% maltose and 10 mM MgSO_4 , was infected with $\lambda\text{placMu53}$ in the presence of helper phage λpMu507 at MOI of 0.1 and 1.0, respectively. The transducing and helper phages were allowed to adsorb for 30 minutes at 37°C without shaking. Following phage adsorption, the bacteria were pelleted by centrifugation at $2,800 \times g$ for 5 minutes at room temperature and washed three times in LB. The final bacterial pellets were suspended in 1 ml of PBS. Lactose fermenting (Lac^+), kanamycin resistant (Km^R) transductants were selected by plating the transduction mixtures at 37°C on M9 salts minimal medium (Davis *et al.*, 1980) supplemented with lactose, kanamycin, and nicotinic acid. Resultant transductants were patched in duplicate on the selective medium to screen for temperature-regulated lactose utilization which was assessed by growth at 37°C and no growth at 30°C. Mutants which expressed a temperature-regulated Lac^+ phenotype were screened for virulence in the HeLa cell invasion assay. Temperature-regulated $\lambda\text{placMu53}$ operon fusions were then transduced into the 2457T

background by generalized transduction using bacteriophage Plvir as described above. Lac⁺, Km^R transductants were detected on LB agar medium containing kanamycin and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (Xgal, [Sigma]; 30 μ g/ml) and characterized for alteration of their virulence phenotypes in the Hela cell invasion assay as well as the other virulence assays described above.

Plasmid preparation and agarose gel electrophoresis. Rapid plasmid screening of 1.5 ml overnight cultures followed the alkaline lysis procedure (Birnboim and Doly, 1979). Briefly, overnight cultures grown at 37°C with aeration were pelleted by centrifugation at 5,500 x g for 1 minute at room temperature. Bacterial pellets were suspended in 100 μ l of pre-lysis buffer (25 mM Tris hydrochloride, 10 mM EDTA, 50 mM glucose) by vigorous vortexing and then lysed by mixing for 5 minutes at room temperature in 200 μ l of lysis solution (0.2 N NaOH, 1% SDS). Chromosomal DNA was precipitated and RNA was removed by neutralization of the lysis mixture with the addition of 1/10 volume of 3 M sodium acetate (pH 5.2), followed by mixing of the samples by inversion for 5 minutes at room temperature. The plasmid lysates were then cleared by centrifugation at 5,500 x g for 5 minutes at 4°C and the supernatants retained. Contaminating cellular RNA was digested for 30 minutes at 37°C with RNaseH (Sigma) at a final enzyme concentration of 100 μ g/ml. Contaminating proteins were then removed by phenol-chloroform-isoamyl alcohol (25:24:1; vol/vol/vol) extraction, followed by chloroform-isoamyl alcohol (24:1; vol/vol) extraction of the phenol (Maniatis *et al.*, 1982). The plasmid

DNA was then precipitated with the addition of two volumes of cold 100% ethanol and pelleted by centrifugation at 10,000 x *g* for 10 minutes at 4°C. DNA pellets were dried by vacuum desiccation and suspended in deionized H₂O. Large scale plasmid DNA isolation was performed by either the same method followed by further purification of plasmid DNA by cesium chloride-ethidium bromide density gradient ultracentrifugation or by the Circleprep™ kit (Bio101, La Jolla, CA). The Circleprep technique followed the manufacturer's specified protocol with the addition of a RNase step as described above following LiCl precipitation of high molecular weight RNA.

DNA samples were screened by electrophoresis in horizontal 0.7 to 1.0% agarose (Sigma) gels in TAE buffer (40 mM Tris acetate, 1 mM EDTA; Maniatis *et al.*, 1982). Preparative electrophoresis of DNA was performed in 0.75 to 1.5% SeaPlaque™ GTG low melt agarose (FMC Bioproducts, Rockland, ME) under the same electrophoretic conditions described above.

Preparation of chromosomal DNA. Chromosomal DNA was prepared from 1.5 ml saturated overnight TSB cultures grown at 37°C as described (Ausubel *et al.*, 1987). Bacteria were pelleted by centrifugation at 5,500 x *g* for 2 minutes at room temperature and lysed in 600 µl of T.E. (10 mM Tris hydrochloride, 5 mM EDTA, [pH 8.0]) containing 0.5% SDS and 100 µg/ml of proteinase K (Sigma) for 1 hour at 37°C. To facilitate precipitation of cell wall debris, polysaccharides, and remaining proteins, the lysis solution was thoroughly mixed with 100 µl of 5 M NaCl followed by the addition of 80 µl of CTAB/NaCl (10% hexadecyltrimethyl ammonium bromide, 4.1% NaCl) and incubated for 10 minutes at 65°C. CTAB-protein-

polysaccharide complexes were then removed by extracting the solution with an equal volume of chloroform-isoamyl alcohol (24:1; vol/vol) followed by centrifugation of the mixture at 5,500 x g for 5 minutes at room temperature. Any proteins remaining in the aqueous phase were extracted with an equal volume of phenol-choloroform-isoamyl alcohol (25:24:1). The organic and aqueous phases were separated by centrifugation and the chromosomal DNA, contained in the aqueous phase, was precipitated with the addition of 0.6 volumes of isopropanol. The DNA was pelleted by centrifugation at 5,500 x g for 10 minutes at room temperature. DNA pellets were washed with 70% ethanol, vacuum desiccated and the final pellets were suspended in 100 μ l sterile deionized H₂O.

Preparation of probe DNA. DNA probes were prepared by restriction digestion of plasmid DNA followed by isolation and purification of DNA fragments from agarose gels by electroelution (Elutrap, Schleicher and Schuell Inc., Keene, NH) or Glassmilk extraction ("Geneclean" kit; Biol01). Whole plasmid or restriction fragment probes were labeled with ³²P by random primer labeling (Pharmacia LKB Biotechnology Inc, Piscataway, N.J.). Briefly, DNA to be labeled was diluted to a final concentration of 75 ng/ μ l in T.E. then denatured by heating to 95 to 100°C for 2 to 3 minutes. Samples were immediately placed on ice for 2 minutes. The denatured template was then mixed with "Reagent mix", [α -³²P] dCTP, and Klenow fragment (5-10 units) provided in the kit and incubated for 30 to 60 minutes at 37°C. Unincorporated [α -³²P]dCTP was removed from the sample by two passages over a Sephadex G-50 resin column (DNA Grade; Bio-Rad). The amount of incorporated label was then determined by assessing the

number of radioactive counts per minute (cpm). For the purpose of Southern hybridization, 3×10^6 cpm of labelled probe DNA was base denatured with NaOH (0.1 N final concentration) for 10 minutes followed by neutralization of the base with 0.18 M Tris hydrochloride - 20 mM Tris base.

Southern hybridization. DNA fragments to be hybridized were electrophoresed as described above. DNA transfer and hybridizations were carried out by the method of Southern (Southern, 1975). Prior to DNA transfer and immobilization on nitrocellulose membranes, the DNA was hydrolyzed and denatured. DNA was partially hydrolyzed by acid depurination to facilitate transfer of large DNA fragments. Agarose gels were soaked twice in 0.25 M HCl at room temperature for 10 to 15 minutes. Gels were rinsed twice with deionized H₂O followed by denaturation of the DNA by soaking gels twice at room temperature for 15 minutes in 0.5 N NaOH - 1.0 M NaCl. Gels were again rinsed with deionized H₂O before being neutralized twice for 30 minutes in several volumes of 0.5 M Tris hydrochloride - 1.5 M NaCl. Gels were again rinsed with deionized H₂O before transferring the DNA to nitrocellulose filters (Bio-Rad) by capillary blot using 10 X SSC (1.5 M NaCl, 0.15 M sodium citrate) as the transfer buffer. Following DNA transfer, the nitrocellulose filters were washed for 20 minutes in 2X SSC before being air dried. DNA fragments were immobilized on the filter by baking under vacuum for 2 to 3 hrs at 80°C. The nitrocellulose filter was soaked in 6X SSC for 2 minutes before pre-hybridization. The pre-hybridization and hybridization steps were carried out in a Hybridease cassette (Hoeffer, San Francisco, CA).

Nitrocellulose membranes were pre-hybridized by soaking in a solution containing 6X SSC, 0.5% SDS, 5X Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin [pentax fraction V]), and 100 mg/ml salmon sperm DNA (Sigma) for 2 to 12 hours at 56°C. Following removal of pre-hybridization solution the filters were hybridized in a solution containing 6X SSC, 0.5% SDS, 10 mM EDTA, 5X Denhardt's, 100 mg/ml salmon sperm DNA, and 3×10^6 cpm of labelled and denatured probe DNA for 2 to 12 hours at 56°C. Filters were washed once in 2X SSC containing 0.2% SDS followed by a second wash at 50°C for 30 minutes and a third wash at 50°C for 1 hour. After the final wash step, the blots were air dried at room temperature and the filters were exposed with Kodak XAR-5 film (Eastman Kodak, Rochester, N.Y.) overnight at -70°C.

β -galactosidase assay. β -galactosidase activity was measured by the method of Miller (Miller, 1972). Bacterial cultures were grown overnight at 30°C or 37°C in TSB and then subcultured at a 1:100 dilution at the same temperatures and allowed to grow approximately four to five generations ($\text{O.D.}_{600\text{nm}} = 0.4$ to 0.6) before assaying for β -galactosidase. Antibiotics were added to the medium for maintenance of the *inv::lacZ* operon fusions, transposon insertions, and recombinant plasmid DNA in both overnight cultures and subcultures. After reaching the appropriate culture density, the subcultures were immediately chilled on ice to inhibit bacterial growth before continuation of the enzyme assay which was performed at room temperature. The optical density of the bacterial cultures at 600 nm was assessed spectrophotometrically before permeabilization of the cultures with chloroform and SDS (10% and 0.005%

final concentration). A volume of 0.1 or 0.5 ml of the permeabilized cultures was diluted in Z-buffer (60 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 40 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 10 mM KCl, 1 mM $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$, 50 mM β -mercaptoethanol) to a final volume of 1.0 ml. The reaction was then started by the addition of 0.2 ml of O-nitrophenyl- β -D-galactopyranoside (ONPG; 4 mg/ml stock solution; Sigma). Completed reactions were stopped by the addition of 0.5 ml of 1 M sodium carbonate. The optical density of each reaction was then assessed spectrophotometrically at 420 nm and 550 nm and the relative amount of β -galactosidase was expressed in Miller units (Miller, 1972) as determined by the following formula:

$$\text{Miller units} = 1000 \times \frac{\text{O.D.}_{420\text{nm}} - (1.75 \times \text{O.D.}_{550\text{nm}})}{T \times V \times \text{O.D.}_{600\text{nm}}}$$

Where T represents the time of the reaction in minutes and V represents the volume of permeabilized bacteria used in the reaction.

DNA ligation. All DNA ligations used in the cloning procedures described in this study were performed using bacteriophage T4 DNA ligase. Ligations were performed according to procedures previously described (Maniatis *et al.*, 1982) in the presence of 1 X ligation buffer (500 mM Tris hydrochloride [pH 7.4], 100 mM MgCl_2 , 100 mM dithiothreitol, 10 mM spermidine), 10 mM ATP and 0.1 to 1 Weiss unit of ligase, at 14°C for 12 to 14 hours. The ligase was inactivated by heating the ligation reactions to 75°C for 10 minutes. Ligations using DNA isolated in agarose gel slices were performed under the same conditions. Following heat inactivation, the ligations were placed at 37°C to prevent solidification

of the agarose and facilitate sample removal for transformation.

Addition of synthetic oligonucleotide linkers. Restriction site specific oligonucleotide linkers were added to insert DNA according to procedures previously described (Ausubel *et al.*, 1987) to facilitate the ligation of DNA fragments with incompatible ends. The plasmid DNA serving as the source of insert DNA was digested with the appropriate restriction enzymes which resulted in DNA fragments with either 5' or 3' overhang ends. DNA fragments with 5' overhangs were converted to blunt ends by the addition of the four deoxynucleotide triphosphates, dATP, dGTP, dCTP, and dTTP, at a concentration of 0.5 mM each in the presence of 1 to 5 units of Klenow fragment at 30°C for 15 minutes. DNA fragments with 3' overhang ends were blunt-ended by the addition of dATP, dGTP, dCTP, and dTTP, at a concentration of 0.1 mM each, 10X T4 DNA polymerase buffer (500 mM Tris hydrochloride, 50 mM MgCl₂, 50 mM dithiothreitol, 500 µg/ml BSA [molecular biology grade; Promega, Madison, WI]) and 0.5 U T4 DNA polymerase (Promega) per µl at 37°C for 20 minutes. Both the Klenow fragment and T4 DNA polymerase were then inactivated by heating the samples to 75°C for 10 minutes. The DNA was purified by the GeneClean technique to remove any remaining enzyme. Phosphorylated linkers (Pharmacia LKB) in the amount of 1 µg were ligated to the blunt-ended molecules as described above. Following ligation of the oligonucleotides, the DNA was again purified before restriction digest of the linker DNA with the appropriate restriction endonuclease. The excess linker molecules which resulted from the restriction digest were then separated from the DNA sample by preparative agarose gel electrophoretic isolation of the DNA fragment of

interest. The DNA fragment generated by the addition of restriction site linkers was then ligated to appropriately digested vector DNA as described above.

Cloning of *inv::lacZ* fusion end joints. Each of the *inv::lacZ* fusions was cloned using the *lacZ* fusion cloning vector pMLB524 (Berman *et al.*, 1984). Plasmid DNA prepared from each *inv::lacZ* fusion mutant was digested with *EcoRI* and ligated to *EcoRI* digested and calf intestinal alkaline phosphatase (CIP) treated pMLB524. Cloned DNA was transformed into *E. coli* strain DH5 α and positive clones were selected on medium containing ampicillin and Xgal.

Cosmid cloning. Construction of the C600 chromosomal library using cosmid cloning vector pCVD301 followed procedures described by Maniatis *et al.* (Maniatis *et al.*, 1984). High molecular weight C600 chromosomal DNA was isolated and partially digested with restriction enzyme *Sau3A*. Partially digested DNA was then fractionated on a 10-30% continuous sucrose gradient to enrich for fragments in the size range of 20-30 kb. These fragments were then ligated into cosmid cloning vector pCVD301 which had been digested with *BamHI* and treated with CIP. In vitro packaging of ligated DNA into bacteriophage λ heads followed methods provided in "Gigapack" in vitro packaging kit (Stratagene Cloning Systems, San Diego, CA).

Isolation of nested deletions. Unidirectional deletions of cloned DNA were generated according to the double stranded Nested Deletion Kit (Pharmacia LKB Biotechnology) protocol, with some modifications. Briefly,

5 to 10 μ g of pAEH106 were linearized by restriction endonuclease digestion with *Kpn*I and *Bam*HI. These restriction sites were located in the cloning vector portion of the recombinant plasmid DNA and were selected to ensure the unidirectional nature of the exonuclease activity. The linearized DNA was then precipitated with the addition of cold 100% ethanol, pelleted by centrifugation at 5,500 x g at 4°C for 10 minutes and the DNA pellet was suspended in 2X ExoIII buffer containing 75 mM NaCl as provided by the manufacturer. Based on the manufacturer's specified protocol, the deletion reactions were then performed in the presence of 100 units of exonuclease III at 25°C and samples were removed from the reaction every 5 minutes and placed on ice. Under these conditions, each time point represented digestion of an additional 300 bp of insert DNA. Following the removal of the last sample time point, each sample was digested with S1 nuclease (0.5 units) for 30 minutes at room temperature to ensure the formation of blunt-ended fragments of DNA. The nuclease reaction was stopped by the addition of S1 stop buffer provided by the manufacturer, followed by heating of the samples to 65°C to inactivate the enzyme. The extent of deletion represented by each sample was verified by agarose gel electrophoresis. Each representative deletion sample was then subjected to preparative agarose gel electrophoresis followed by excision of the DNA fragment from the gel. The agarose plug was melted by heating to 65°C for 10 minutes and 15 μ l of the sample was ligated on itself in the presence of 10 Weiss units of ligase. Following the intramolecular ligation of the nested deletion plasmid DNA species, 5 μ l of each reaction was used to transform *E. coli* strain DH5 α . Plasmid DNA was then purified from five representative transformants from each sample

transformation and analyzed to verify the presence of a recombinant plasmid containing the appropriate size deletion. Positive nested deletion clones were then retained for nucleotide sequence analysis.

DNA Sequencing. Nucleotide sequence determination of the 1.8 kb and 2.3 kb *EcoRI*-*AccI* fragments represented by clones pAEH122 and pAEH104, respectively, was performed on double stranded DNA templates purified by the Circle prep technique described above. In addition, the 2.3 kb fragment was partially sequenced from the nested deletion clones of pAEH106. 15-mer or 17-mer oligomer primers (Central Resource Facility, USUHS) were used to prime dideoxy chain termination reactions on the templates using the Sequenase Version 2.1 DNA sequencing kit (US Biochemical Corporation, Cleveland OH). The double stranded DNA sequencing protocol provided by the manufacturer was used with some modifications. The plasmid DNA to be sequenced (5 to 6 μ g) was base denatured with 1.0 N NaOH at a final concentration of 0.2 N for 5 minutes at room temperature. Oligonucleotide primer was annealed to the template DNA by mixing 5 ng of the primer oligonucleotide with the denatured template DNA for 30 seconds at room temperature. The annealed DNA was precipitated by the addition of 3 M sodium acetate (pH 5.2) to a final concentration of 0.2 M and two volumes of cold ethanol. The DNA was pelleted by centrifugation at 10,000 x g for 10 minutes at 4°C. The pellet was washed once with 70% ethanol and vacuum desiccated before being suspended in 1 X Sequenase bufferTM. The labelling and termination reactions followed those specified by the manufacturer. Completed sequencing reactions were then electrophoresed on vertical, wedge-spacer

7.2% polyacrylamide gels containing 46% urea, for approximately 6 hours at 80 watts constant power in TBE (89 mM Tris borate, 2 mM EDTA). Gels were then immersed in a solution of 10% methanol - 10% acetic acid to remove the urea prior to being dried and exposed to Kodak XAR-5 film (Eastman Kodak) at room temperature for 12 to 24 hours. Analysis of nucleotide sequence was performed using the Genetics Computer Group Version 6.2 sequence analysis software package (Devereux *et al.*, 1984).

RESULTS

Part I: Isolation and characterization of temperature-regulated virulence genes.

Isolation and initial characterization of temperature-regulated *lacZ* operon fusions. The invasive *S. flexneri* strain BS181 was infected with bacteriophage λ placMu53 and lysogens expressing Km^R , the selective marker encoded by the transposing phage, were selected for the ability to utilize lactose (Lac^+) at 37°C. Approximately 10,000 Lac^+ Km^R lysogens were subsequently screened for the Lac^+ phenotype at 37°C and 30°C. Of the Lac^+ mutants screened, seven independently isolated mutants were temperature-regulated for lactose utilization since they exhibited a Lac^+ phenotype only at 37°C. The actual levels of β -galactosidase expressed by these mutants at 37°C and 30°C was determined and, in each case, the level of expression at 37°C was at least 5-fold greater than the levels expressed at 30°C (data not shown). Having established that the seven fusion mutants isolated were the result of *lacZ* fusions to temperature-regulated genes, each mutant was tested for expression of virulence in the HeLa cell invasion assay. Four of the seven mutants exhibited a complete loss of invasive ability (Inv^-) while the other three expressed an invasion phenotype equivalent to the parental strain, BS181. Therefore, preliminary characterization of the temperature-regulated *lacZ* fusion mutants indicated, as previously shown (Maurelli and Curtiss, 1984), that two separate populations of *lacZ* operon fusions could be isolated: one in which the genes marked by the fusion were involved in HeLa cell invasion

(*inv::lacZ*) and a second in which the invasion phenotype was unaltered.

To further characterize the operon fusion mutations, each fusion was independently transduced into the non-mutagenized wild-type *S. flexneri* background (2457T). The resultant Lac⁺ Km^R transductants were tested both for temperature-regulated expression of β -galactosidase and for the ability to invade HeLa cells. As shown in Table 3, each transductant tested expressed the same phenotype as the original operon fusion mutant from which it was derived. Fusion strains BS226, BS228, BS230, and BS232 were Inv⁻ while fusion mutants BS234, BS236, and BS238 remained Inv⁺. All 7 mutants expressed a 37°C to 30°C β -galactosidase ratio greater than or equal to 3. These results confirmed that the original mutant strains were each the result of a single site insertion of the transposing bacteriophage and that the non-invasive mutants identified genes which were required for invasion. In addition, these data demonstrated that the two populations of fusions isolated were also different in their relative expression of β -galactosidase at 37°C and 30°C. Each non-invasive operon fusion mutant showed a 37°C to 30°C β -galactosidase ratio greater than 40. In marked contrast, the mutants which remained invasive all expressed β -galactosidase ratios of less than 10 (Table 4).

Phenotypic characterization of invasive *lacZ* operon fusion mutants. (i)

Plaque and Séreny assays. The HeLa cell invasion assay defines only one step in *Shigella* pathogenicity. To determine if another step in *Shigella* virulence may have been altered as a result of mutation by λ placMu53, the three Inv⁺ operon fusion isolates were screened for virulence as measured

TABLE 3. Virulence phenotypes of temperature-regulated *lacZ* operon fusion mutants.

Strains	HeLa cell invasion ^a	Plaque assay ^b	Sereny test ^c	Contact hemolysis (%) ^d	Congo red binding 30°C	Congo red binding 37°C	Regulation by <i>virR1</i> ^e
BS226	-	ND ^f	ND	3	-	-	+
BS228	-	< 1 x 10 ⁻⁸	-	7	-	+	+
BS230	-	ND	ND	9	-	-	+
BS232	-	ND	ND	15	-	-	+
BS234	++++	7.2 x 10 ⁻⁴	+	80	-	+	+
BS236	++++	6.2 x 10 ⁻⁴	+	106	-	+	-
BS238	++++	5.3 x 10 ⁻⁴	+	104	-	+	+
2457T	++++	6.3 x 10 ⁻⁴	+	100	-	+	NA ^g
BS103	-	< 1 x 10 ⁻⁸	+	16	-	+	NA

^a Data represent percentage of HeLa cells invaded: (number of HeLa cells invaded/total number HeLa cells counted) x 100. +++, 77 to 100%; +, 54 to 76%; ++, 31 to 53%; +, 10 to 30%; -, <10%. Cells containing >5 bacteria were considered invaded.

^b Plaqueing efficiency expressed as number of plaques per input CFU.

^c Positive Sereny test was determined to be elicitation of keratoconjunctivitis 48 h postinoculation.

^d Values represent percentage of mean absorbance obtained for wild-type strain 2457T

^e See Table 4 for β -galactosidase 37°C/30°C ratios.

^f ND, not determined.

^g NA, not applicable

TABLE 4. Expression of β -galactosidase by *lacZ* operon fusion mutants and their *virR::Tn10* derivatives^a.

Strain / Fusion #	U of β -galactosidase ^b		Ratio 37°C/30°C	<i>virR::Tn10</i> derivatives		
	37°C	30°C		Units of β -galactosidase ^b	30°C	Ratio 37°C/30°C
BS226 / 11.5	550	11	50	1,080	580	1.9
BS228 / 17.6	382	6.3	60	840	210	4.0
BS230 / 17.7	668	14	49	1,730	560	3.0
BS232 / 18.15	468	4.4	106	1,390	440	3.2
BS234 / 17.9	132	43	3	98	204	0.5
BS236 / 18.11	194	40	5	312	104	3.0
BS238 / 18.13	338	54	6	180	160	1.1

^a Values presented are based on representative experiments

^b Units of β -galactosidase are as defined by Miller (Miller, 1972)

by the plaque and Séreny assays. The results (Table 3) indicate that the fusion mutants are identical to the wild-type strain 2457T in their ability to form plaques and cause a positive Séreny test. Keratoconjunctivitis elicited by the fusion mutants and 2457T developed after 48 hrs and were equivalent in intensity.

(ii) Ability of *lacZ* operon fusion mutants to bind Congo red dye. Temperature-regulated binding of Congo red is characteristic of virulent *Shigella* strains and loss of Congo red binding (Pcr^-) is accompanied by loss of invasive ability (Maurelli *et al.*, 1984b). Thus, the seven operon fusion mutants were plated on medium containing Congo red dye and incubated at 37°C and 30°C to determine the effect of the *lacZ* operon fusions on Congo red binding. All of the invasive mutants as well as one of the non-invasive mutants, BS228, exhibited a temperature-regulated Pcr^+ phenotype and colonial morphology identical to that of 2457T. The Inv^- mutants BS226, BS230, and BS232 (the *inv::lacZ* fusion class) exhibited a Pcr^- phenotype at both 37°C and 30°C corresponding to the Pcr phenotype of Inv^- strains (Table 3).

(iii) Contact hemolytic activity (Hly). Although *S. flexneri* classically is defined as non-hemolytic, the organism has been shown to lyse sheep red blood cells (SRBC) when in close contact with the red blood cells (Clerc *et al.*, 1986). This phenotype has been shown to strongly correlate with several different virulence phenotypes, notably invasion. The *lacZ* fusion mutants were tested for contact hemolytic activity relative to the parental strain 2457T and its invasion plasmid cured derivative BS103. As shown in Table 3, the *inv::lacZ* fusion mutants all

expressed an Hly⁻ phenotype. The percentage of Hly activity of the *inv::lacZ* mutants was less than or equal to 15% of the Hly activity expressed by wild-type strain 2457T and comparable to that of the Hly⁻ control strain BS103. In contrast, the Inv⁺ fusion mutants were as efficient in their ability to induce contact hemolysis as 2457T.

virR control of temperature-regulated *lacZ* operon fusions. *virR* has been identified as a gene required for temperature-dependent regulation of *Shigella* virulence (Maurelli and Sansonetti, 1988). To determine if any of the temperature-regulated fusion insertions were located within genes regulated by *virR*, each of the *lacZ* operon fusion mutants was screened for loss of temperature-regulated β -galactosidase expression in the presence of a *virR::Tn10* mutation. A Plvir lysate made on *S. flexneri* strain BS189 was used to transduce a *virR::Tn10* mutation into the *lacZ* operon fusion mutants. The ratios of β -galactosidase activity at 37°C vs. 30°C in the *inv::lacZ* strains (BS226, BS228, BS230, and BS232) were markedly reduced after introduction of the *virR* mutation (Table 4). In the presence of the wild-type *virR*, each one of these Inv⁻ strains had a 37°C to 30°C β -galactosidase ratio of greater than 40 while in the presence of a *virR::Tn10* mutation the ratio was reduced to a value of less than or equal to 5. Decreased β -galactosidase ratios were primarily due to increased enzyme levels at 30°C. Therefore, these data indicate that β -galactosidase expression in the *inv::lacZ virR::Tn10* strains was no longer temperature-regulated. Fusion mutants BS234 and BS238 exhibited a similar reduction of the 37°C to 30°C β -galactosidase ratio. Ratio

values of 3 (BS234) and 6 (BS238) decreased to ratio values of less than 1 when a *virR* mutation was present. Fusion mutant BS236 however, showed no significant reduction in the β -galactosidase ratio in the presence of a *virR* mutation.

Localization of the temperature-regulated *inv::lacZ* operon fusion mutants

(i) Western blot analysis. Random insertion of λ placMu53 in the mutant strains may have resulted in the *inv::lacZ* operon fusions being located on either the chromosome or the 220 kb invasion plasmid. If insertion occurred in the 220 kb invasion plasmid, the presence of the λ placMu53 genome should be detectable by agarose gel electrophoresis, since its large size (approximately 50 kb) would retard the migration of the plasmid in the gel. The first step in localizing the temperature-regulated *inv::lacZ* fusions involved a comparison of the plasmid profiles of the *inv::lacZ* operon fusion strains with that of 2457T. The 220 kb invasion plasmid in the four non-invasive operon fusion mutants was larger in size when compared to the wild-type pSf2a140 (data not shown). These results indicated that each one of the *inv::lacZ* operon fusions was located on the 220 kb invasion plasmid. Use of this same technique revealed that each of the *Inv*⁺ operon fusions also mapped to the invasion plasmid. Further mapping of these *Inv*⁺ fusions was not pursued.

Western blot analysis was used to more precisely locate the *Inv*⁻ fusions on the virulence plasmid. Previous studies have shown that a 37 kb fragment of the 220 kb invasion plasmid defined by cosmid clone pHS4108 (Fig. 5) is the minimum sequence necessary to confer an *Inv*⁺ phenotype on

an invasion plasmidless derivative of *Shigella* (Maurelli *et al.*, 1985). In addition, pHS4108 contains the *ipa* (invasion plasmid antigen) genes. They encode the four major antigenic polypeptides, IpaA (70 kDa), IpaB (62 kDa), IpaC (43 kDa), and IpaD (32 kDa) which are detected by convalescent sera from both humans and monkeys following a *Shigella* infection (Hale *et al.*, 1985; Oaks *et al.*, 1986). Moreover, Tn5 insertions in various regions of pHS4108, including the *ipa* gene cluster, lead to a knock out or reduction of the invasion phenotype (Baudry *et al.*, 1987). To localize the *inv::lacZ* operon fusions on pSf2a140 and determine if the fusions inactivated any one of the *ipa* genes, the operon fusion mutants were analyzed by Western blot using both IpaA, B, C, and D specific polyclonal sera and MAbs specific for either IpaB or IpaC. Fusion mutant BS226, was identical to 2457T in its Ipa protein profile (Fig. 6, Panel A, lane 2), as were fusion mutants BS230 and BS232 (data not shown). Fusion mutant BS228, however, had an altered Ipa protein profile with the bands representing IpaA, C, and D no longer being visible and the band migrating at the same position as IpaB having an increased staining intensity. These findings suggest that of the four *inv::lacZ* operon fusion mutants isolated, only mutant BS228 had an operon fusion insertion located in the *ipa* coding region.

The isolation and characterization of MAbs to IpaB and C (Mills *et al.*, 1988) provided the opportunity to further characterize the location of the *lacZ* operon fusion in strain BS228. Western blot analysis using the IpaC-specific MAb, 5H1, showed no detectable IpaC production by BS228

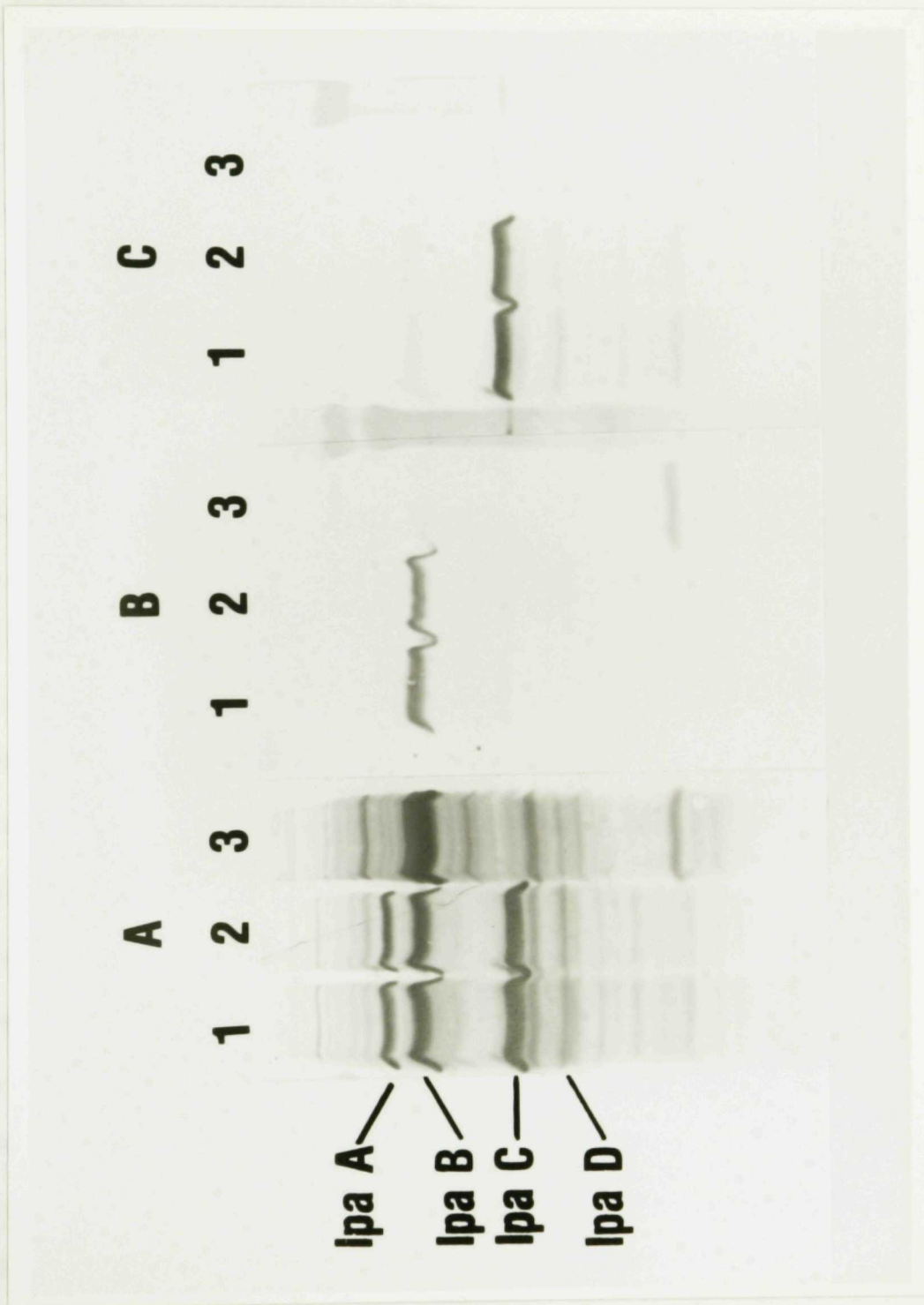
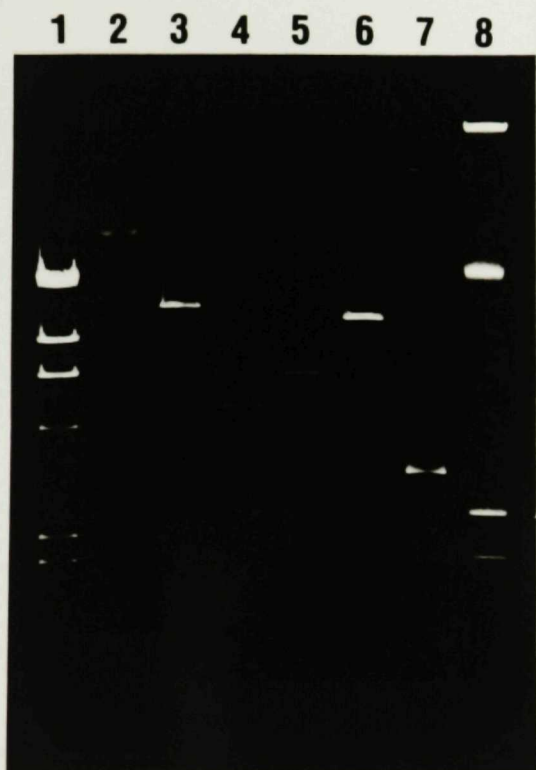
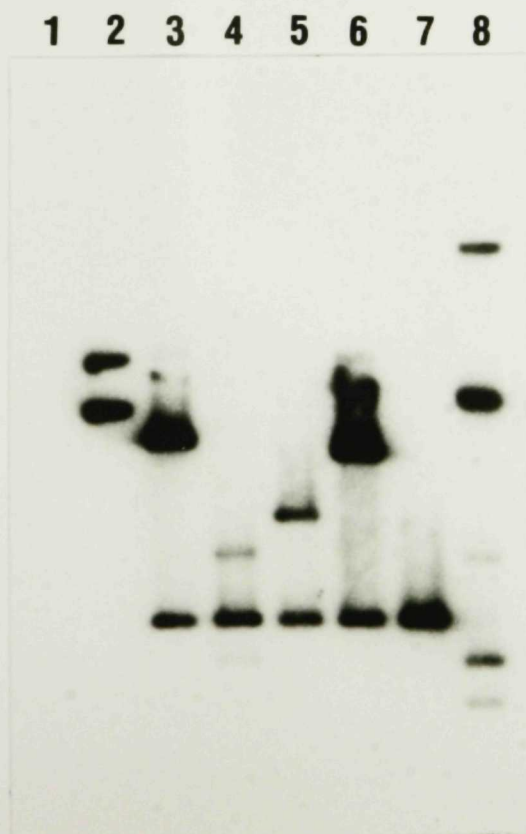


Figure 6. Ipa protein profile of *inv::lacZ* mutants. Western blot of whole-cell bacterial lysates probed with convalescent-phase human polyclonal antisera (1:100 dilution) from a patient infected with *S. flexneri* 2a (A), MAb 2F1 (1:300 dilution) (B), or MAb 5H1 (1:300 dilution) (C). Lanes: 1, 2457T; 2, BS226; BS230; BS232; 3, BS228.

(Fig. 6 Panel C). In contrast, three different IpaB-specific MABs (2F1, 1H4 and 4C8) detected a protein of considerably smaller molecular weight than that of wild-type IpaB in the BS228 lysate (represented by Fig. 6, Panel B). These results are evidence that the *lacZ* operon fusion in BS228 is located in the *ipaB* coding region and, as a result of the insertion, the strain synthesized a truncated IpaB product. Recognition of the truncated IpaB by the MABs established that the fusion insertion was located toward the 5' end of *ipaB* since the MAb epitopes map to the amino-terminal end of IpaB (Mills *et al.*, 1988).

(ii) Southern hybridization. Four subclones spanning the entire length of pHS4108 were used as probes to map the location of the *inv::lacZ* fusions by Southern hybridization (Fig. 7) The *EcoRI* fragments which contain the fusion end joints of the different *inv::lacZ* fusions were first cloned into the *lacZ* fusion cloning vector pMLB524 (Berman *et al.*, 1984). pMLB524 contains 309 bp of the *lac* operon starting at the unique *EcoRI* site in *lacZ* and encodes the 17 carboxy terminal amino acids of β -galactosidase. The plasmid alone exhibits a LacZ⁻ phenotype. By cloning *EcoRI* fragments from a *lacZ* fusion into this vector, the *lacZ* gene can be regenerated and a LacZ⁺ phenotype results. The bacterial DNA represented in the cloned insert includes bacterial DNA from the fusion end joint to the next *EcoRI* site 5' to *lacZ*. *EcoRI* digestion of each *inv::lacZ* fusion recombinant generated two fragments, one which represented pMLB524 and the other the *EcoRI* fusion end joint insert (Fig. 7 Panel A). The size of the cloned *EcoRI* fragments from each of the *inv::lacZ* fusions ranged in size from 5 - 14 kb. After subtracting the 3 kb which account for the *lacZ*

Figure 7. Southern hybridization mapping of *inv::lacZ* fusion mutants. (A) Ethidium bromide stained 0.7% agarose gel; (B, C, and D) autoradiograms of Southern hybridization of gel in panel A using pHS5101 (B), pHS5103 (C), and pHS4011 *Eco*RI fragment (D) as probes. Lanes: 1, Molecular weight markers; 2, probe DNA; 3, *Eco*RI-digested pAEH006 (fusion 11.5); 4, *Eco*RI-digested pAEH007 (fusion 17.6); 5, *Eco*RI-digested pAEH008 (fusion 17.7); 6, *Eco*RI-digested pAEH009 (fusion 18.15); 7, *Eco*RI-digested pMLB524; 8, undigested 2457T plasmid DNA.

A**B****C****D**

portion of the cloned fragments, the bacterial DNA represented in the subcloned fragments had the following sizes: fusion 11.5 (BS226), 10.3 kb; fusion 17.6 (BS228), 2.0 kb; fusion 17.7 (BS230), 4.0 kb; and fusion 18.15 (BS232), 7.0 kb. Southern hybridization using the pHS4108 subclones (Fig. 5) as probes, indicated that each one of the fusion end joint clones hybridized to pHS5103 (Fig. 7 Panel C). The *EcoRI* insert fragment of the fusion 11.5 subclone also hybridized to pHS5101 (Fig. 7 Panel B) whereas none of the fusion end joint subclones hybridized to pHS5102 or pHS5500 (data not shown). Based on these data, the temperature-regulated *inv::lacZ* fusions were all shown to map to pHS4108 and the location of the *lacZ* fusion in mutant BS228 within *ipaB* (*ipaB::lacZ*⁺ 17.6) corroborated the results obtained by Western blot analysis.

In an attempt to determine more specifically the location of the temperature-regulated *inv::lacZ* fusions 17.7 (BS230) and 18.15 (BS232), the subcloned fusion end joints of each were hybridized with the *EcoRI* fragment from pHS4011 which spans the junction between subclones pHS5101 and pHS5103 (Fig. 5). Both fusion subclones hybridized to the *EcoRI* fragment of pHS4011 (Fig. 7 Panel D) thereby, localizing the fusion end joints for strains BS230 and BS232 to the 8 kb *EcoRI* - *SalI* fragment of pHS4108 (Fig. 5). These results, together with the insert size approximations calculated for the fusion end joint subclones, placed the BS230 and BS232 fusion insertion sites at the positions designated in Figure 5. The hybridization data also indicate that the direction of transcription of the 17.7 and 18.15 fusions is in the opposite direction to that of the *ipa* operon.

Further Southern hybridization analysis of fusion 11.5 (BS226)

revealed that this operon fusion insertion was also located on the 11 kb pHS4011 *EcoRI* insert (Fig. 7 Panel D). A restriction endonuclease map of the 11.5 fusion subclone pAEH006, was constructed and the positions of the *Sall*, *XbaI*, and *HindIII* sites were found to correspond to the sites as originally mapped in pHS4108 (Fig. 8). The five *HindIII* fragments of pAEH006 were designated A through E. On the basis of the cloned fragment size in pAEH006 (10.3 kb) and the Southern hybridization data, the location of the fusion end joint site was predicted to be in fragment A. This prediction was confirmed by Southern hybridization of an *EcoRI*-*HindIII* digest of pAEH006 probed with pHS5101 and pHS5103 (Fig. 9). Both probe DNAs hybridized with pMLB524 since the cloning vectors for each of the recombinants are derived from pBR322. pHS5101 also hybridized to *HindIII* fragments D and E (Fig. 9 panel A), while pHS5103 hybridized with *HindIII* fragments B, C, and D (Fig. 9 panel B.). The lack of hybridization to the 750 bp *EcoRI*-*HindIII* portion of fragment 'A with pHS5103 may have been the result of an insufficient amount of pAEH006 electrophoresed on the original agarose gel. The conclusion from these results is that the 11.5 fusion end joint mapped to a site located approximately 0.5 kb to the left of the *EcoRI* site as indicated (Fig. 5), and the direction of transcription was determined to be the same as that of the fusions in strains BS230 and BS232.

Detection of surface IpaB and IpaC by whole cell ELISA. Previous studies employing the MAbs 2F1 and 2G2 in a whole cell ELISA system led to the conclusion that IpaB and IpaC are exposed on the surface of the bacterium

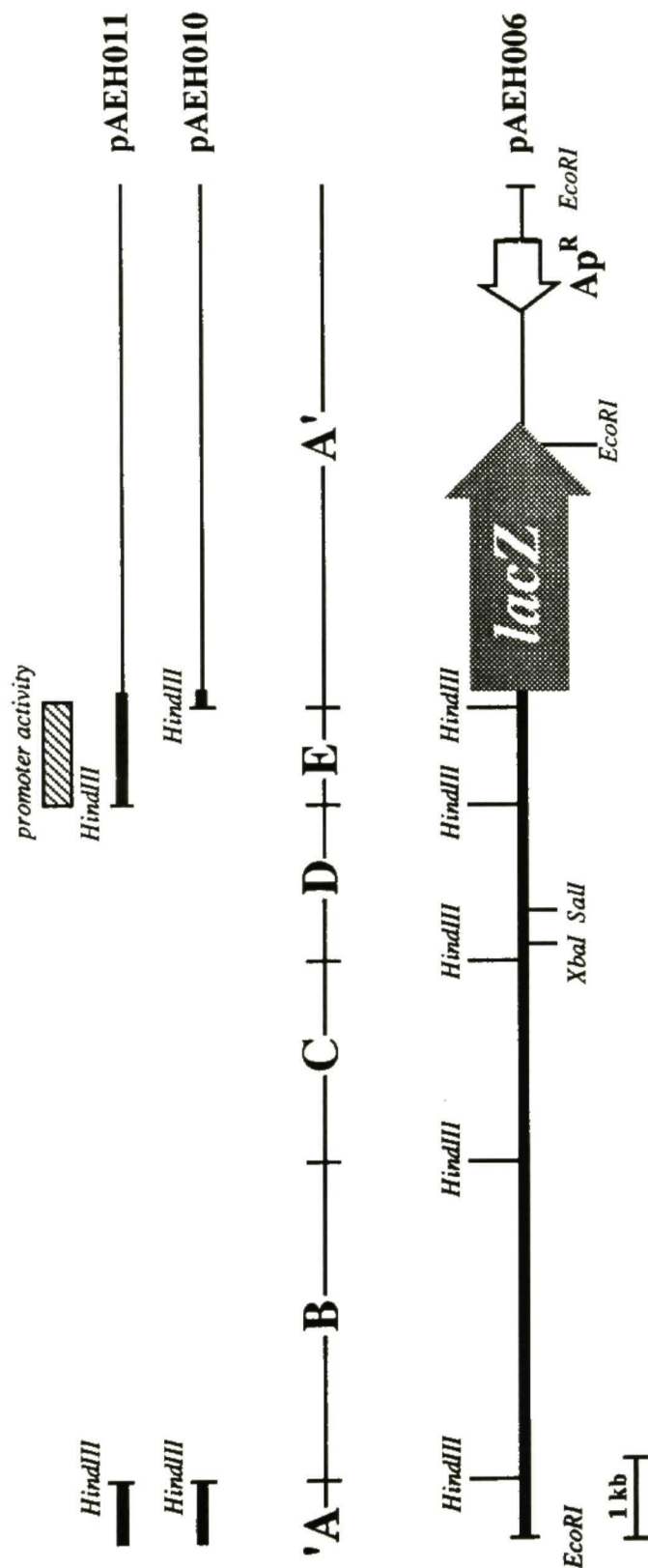


Figure 8. Restriction map of the *mxi::lacZ* fusion end joint subclone pAEH006 and the pAEH006 *Hind*III deletion derivatives. Insert fragment represents 14 kb fragment from *Eco*RI site at left hand end of the plasmid map to the unique *Eco*RI site in the *lacZ* coding sequence. The thick line represents virulence plasmid DNA from the *mxi::lacZ* fusion mutant BS226. The thin line represents cloning vector pMLB524. The *Sal*I site represents the junction between pHS5103 and pHS5101 (Fig. 5). *Hind*III fragments 'A and B are based on *Hind*III sites mapped in pHS5103, while fragments D, E, and A' are based on *Hind*III sites mapped in pHS5101.

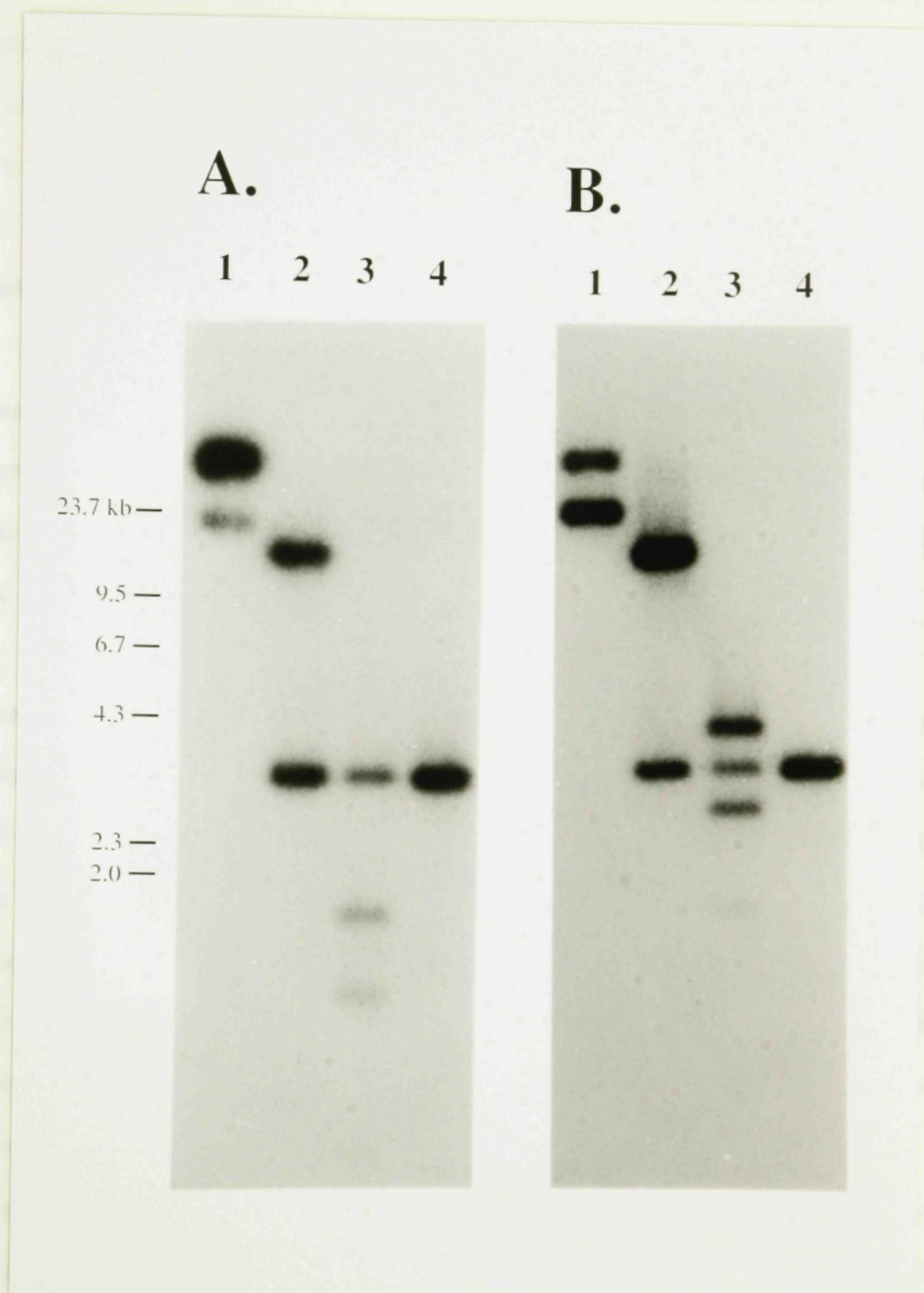


Figure 9. Southern hybridization analysis of *inv::lacZ* fusion subclone pAEH006. Probe DNA used: (A) pH55101 and (B) pH55103. Lanes: 1, uncut probe DNA; 2, *EcoRI* digested pAEH006; 3, *EcoRI-HindIII* digested pAEH006; 4, *EcoRI* digested pMLB524;

(Mills *et al.*, 1988). Western blot data (Fig. 6) indicated that each of the *Inv*⁻ fusion mutants isolated expressed one or all of the Ipa proteins intracellularly. Whole cell ELISA was therefore used to determine if the *inv::lacZ* fusion mutants were altered in the surface expression of IpaB and IpaC.

The results obtained with MAb 2F1 (Table 5) indicated that the expression of the truncated IpaB in BS228 approximated the level of wild-type IpaB expression in the control strain 2457T. The truncated IpaB produced by the fusion mutant appears therefore, to contain sufficient sequence information to be processed and expressed on the bacterial surface. BS226, BS230, and BS232 expressed reduced levels of IpaB on their surface.

The results obtained with MAb 2G2 (Table 5) indicated that mutant strain BS228 expressed a very low level of surface IpaC. This seems to contradict the results of the Western blot analysis which indicated that BS228 was expressing no IpaC. It is possible however, that within the range of sensitivities for these two assay systems, the Western blot may not have detected low level expression of IpaC. BS226, BS230, and BS232 showed intermediate surface expression of IpaC when analyzed with MAb 2G2. The decreased expression of these proteins on the surface of the bacteria may explain the *Inv*⁻ phenotype observed for all 4 of these fusion mutant strains. On the basis of these observations, the genetic loci insertionally inactivated in strains BS226, BS230, and BS232 were designated *mxi* loci (membrane expression of Ipa) and the operon fusion mutations were designated *mxi::lacZ*⁺.

TABLE 5. Expression of surface IpaB and IpaC in *Inv*⁻ temperature-regulated *lacZ* fusion mutants.

Strain	A_{405}^a	
	MAb 2F1 ^b	MAb 2G2 ^c
2457T	1.174 ± 0.104	1.889 ± 0.024
BS226	0.249 ± 0.025	0.700 ± 0.064
BS228	0.882 ± 0.037	0.111 ± 0.003
BS230	0.247 ± 0.035	0.680 ± 0.065
BS232	0.243 ± 0.014	0.613 ± 0.047
BS103 ^d	0.046 ± 0.020	0.048 ± 0.003

^a Representative experimental values expressed as means of quadruplicate samples ± standard deviations of the means. A_{405} values ≤ 0.1 were considered negative.

^b IpaB-specific MAb.

^c IpaC-specific MAb.

^d Negative control strain.

Isolation and characterization of the *virR*-regulated *mxi* promoter from operon fusion 11.5. As described above, *EcoRI* fragments which contain the *lacZ* fusion end joints from each of the *Inv⁻* mutants were cloned into pMLB524. Provided that the *EcoRI* sites 5' to the fusion end joints are far enough upstream of the target genes, these clones may include the target gene promoters. *E. coli* K-12 DH5 α was transformed with the different *mxi::lacZ⁺* and the *ipaB::lacZ* fusion clones and *LacZ⁺* transformants were isolated on agar medium which contained the chromogenic β -galactosidase substrate X-gal. Quantitation of the β -galactosidase produced by the transformants, however, indicated that the enzyme levels at 30 and 37°C deviated substantially from those observed for the parental fusions in the *S. flexneri* background. When compared to the β -galactosidase activity observed for the corresponding operon fusions, the clones produced amounts of β -galactosidase which were 10- to 40-fold lower at 37°C, and 2 to 6 fold higher at 30°C (Table 6). In addition, the β -galactosidase levels produced by each clone were approximately the same at 30 and 37°C. As a result, temperature-regulated β -galactosidase expression was not detected. These results suggest that either the fusion end joint clones did not include the promoter regions of the virulence genes they represent, or that they carried promoters which require *Shigella*-specific sequences for their expression.

To test these possibilities, each fusion end joint clone was transformed into *S. flexneri* strains BS103 and BS201. The rationale for selecting these strains was two-fold. First, the temperature-regulated β -galactosidase activity exhibited by the parental fusions was identified in a *Shigella* genetic background. Second, plasmid encoded transcriptional

TABLE 6. β -galactosidase expression of pMLB524 *inv:lacZ* fusion clones in *E. coli* K-12 and *S. flexneri* 2a strains^a.

Fusion	pMLB524 clone	Units β -galactosidase 37°C / 30°C ^b			
		DH5 α	BS103	BS201	Parental fusion
11.5	pAEH006	55 / 69	63 / 67	456 / 51	550 / 11
17.6	pAEH007	14 / 14	15 / 15	24 / 18	382 / 6.3
17.7	pAEH008	20 / 42	ND ^c	74 / 67	668 / 14
18.15	pAEH009	12 / 16	27 / 15	24 / 25	468 / 4.4

^a DH5 α is an *E. coli* K-12 strain; BS103 and BS201 are *S. flexneri* 2a strains. Values presented are based on representative experiments.

^b Units of β -galactosidase are as defined by Miller (Miller, 1972)

^c Not determined

activators of *Shigella* virulence gene expression have been identified (Adler *et al.*, 1989). Therefore, if the *S. flexneri* transformants exhibit a β -galactosidase phenotype similar to that of the DH5 α transformants, then the absence of a cloned promoter would be confirmed. On the other hand, if transcription from the cloned promoters requires *Shigella*-encoded activators, then the transformants should express β -galactosidase levels comparable to that of the parental fusions. BS103 and BS201 are derivatives of wild-type *S. flexneri* (Table 2). BS103 is cured of the virulence plasmid while BS201 carries the virulence plasmid plus a *recA*⁻ mutation. Thus, these strains permit discrimination between a requirement for chromosomal and/or plasmid sequences in promoter activation.

β -galactosidase levels at 30 and 37°C were measured in transformants of these strains. Only the BS201/pAEH006 transformant exhibited β -galactosidase activity at 37°C comparable to that of its parental fusion. The remaining clones showed levels of β -galactosidase activity at 30 and 37°C, unchanged from those observed for their corresponding DH5 α transformants. At 30°C the levels of β -galactosidase produced by BS201/pAEH006 were approximately 5 times higher than those observed in 11.5 (Table 6). Increased copy number of the temperature-regulated promoter cloned on pAEH006 and in turn insufficient amounts of suppressor activity is a possible explanation for the higher amounts of enzyme observed for the clone at 30°C when compared to the levels of enzyme generated by the parental fusion (11.5). The β -galactosidase activity observed for the promoterless clones is most likely due to transcription initiated from a pMLB524 promoter.

On the basis of the β -galactosidase levels expressed by the BS103

and BS201 transformants, it appears that i) only pAEH006 harbored a temperature-regulated virulence gene promoter, and ii) the *S. flexneri* virulence plasmid was essential for promoter expression. In addition, the inability to clone a promoter for either fusion 17.7 or 18.15, which were both mapped to a location 5' of fusion 11.5, suggests that the promoter activity identified with these fusions is located to the left of the *EcoRI* site shared by pHS4011 and pHS5103 (Fig. 5). Moreover, these data indicate that *mxi::lacZ*⁺ fusion 11.5 represents a transcription unit unique from *mxi::lacZ*⁺ fusions 17.7 and 18.15.

Localization of the temperature-regulated *mxi* promoter on fusion end joint clone pAEH006. To more precisely map the temperature-regulated *mxi* gene promoter, a series of *HindIII* deletion subclones was generated in pAEH006. As illustrated in Figure 8, the absence of *HindIII* sites within *lacZ* facilitated the deletion of DNA sequences upstream of the fusion end joint without eliminating the ability to screen for promoter activity by β -galactosidase activity. Deletion clones were generated by limited digestion of pAEH006 with *HindIII* followed by intra-molecular ligation of the DNA restriction fragments. Previous results indicated that fusions 17.7 and 18.15 represented transcription units independent of fusion 11.5 and mapped to regions of pHS4108 which correspond to *HindIII* fragments C and D (Fig. 8). Only constructs pAEH010 (*HindIII* fragment A) and pAEH011 (*HindIII* fragments A-E) were therefore retained for characterization because they most likely represent regions 3' to the genes marked by fusions 17.7 and 18.15. The two deletion clones were screened for promoter activity by measurement of β -galactosidase levels produced by

BS201/pAEH010 and BS201/pAEH011 transformants. As shown in Table 7, the levels of β -galactosidase produced by BS201/pAEH011 at 37°C approximated the levels expressed by BS201/pAEH006, while the BS201/pAEH010 transformant expressed levels of β -galactosidase at 30 and 37°C comparable to those detected in the DH5 α and BS103 transformants of pAEH006 (Table 6). These results indicate that the temperature-regulated *mxl* gene promoter identified in fusion 11.5 was located either on *Hind*III fragment E or spanned the junction between fragments E and A'. This places the promoter within 2.2 kb of the *mxl::lacZ*⁺ 11.5 fusion end joint (Fig. 8).

Part II: Characterization of *virR*.

Identification of *virR* homolog in *E. coli* K-12. The strategy used to identify a *virR* homolog in *E. coli* K-12 was based on previous studies of *virR* in *S. flexneri* which mapped the gene to a chromosomal locus between *galU* and *trp* (Maurelli and Sansonetti, 1988). A lysate of the generalized transducing bacteriophage Plvir was prepared on *E. coli* K-12 strain C600 and used to transduce chromosomal DNA adjacent to the *E. coli* *trp* operon into *S. flexneri* strain BS214. This strain is deleted for the region spanning *virR* to *trp* and consequently is constitutive for expression of the invasive phenotype. Of the 95 transductants which were selected for restoration to prototrophy (Trp⁺), 10 were chosen at random and scored for temperature regulation of the invasive phenotype in the HeLa cell invasion assay. All 10 Trp⁺ transductants were invasive only after growth at 37°C, which indicated restoration of the wild-type phenotype. The degree of invasion for the transductants, measured as a percent of total HeLa

TABLE 7. β -galactosidase expression from pAEH006 deletion subclones.

Strain	Plasmid	Units β -galactosidase ^a		Ratio
		37°C	30°C	37°C / 30°C
BS201	pAEH010	37	18	2.1
BS201	pAEH011	246	24	10
BS201	pAEH006	502	51	9.8
BS226		447	15	30

^a Units of β -galactosidase expressed are as defined by Miller, (Miller, 1972)

cells counted, was between 50-94%, comparable to those obtained for the wild-type strain 2457T at 37°C (approx. 80%). No invasion was observed when the Trp⁺ transductants were grown at 30°C. This is in contrast with the results obtained with the parent *virR* mutant, BS214, which is invasive at both 37°C and 30°C. Transduction of BS214 with a Plvir lysate made on another *E. coli* K-12 strain, MG1655, yielded identical results in the HeLa cell invasion assay when Trp⁺ transductants were compared to the control strains 2457T and BS214. MG1655, also a non-pathogenic *E. coli* K-12 strain, represents a lineage which is distinct from that of strain C600. The results obtained for the two separate transductions strongly suggest that the region of the *E. coli* K-12 chromosome which corresponds to the region spanning *virR-trp* of the *S. flexneri* chromosome contains a gene(s) homologous in function to *virR* of *Shigella*.

Cloning of *virR* homolog from *E. coli* K-12 strain C600. The strategy for cloning *virR* from *E. coli* exploited the known chromosomal location of *virR* in *Shigella*, specifically, the tight linkage between *galU* and *virR* (Maurelli and Sansonetti, 1988). As described in the Materials and Methods, a C600 chromosomal library was constructed and the cloned DNA was then transduced into *E. coli* strain ATM016 which is deleted for *galU*. GalU⁺ clones were selected by their ability to utilize galactose as a sole carbon source. In three separate transductions, 13 GalU⁺ clones were obtained. Recombinant plasmid DNA from one such GalU⁺ clone (pAEH001) was transformed into *S. flexneri* strain BS255 to determine if the cloned DNA could complement the *virR* defect in this strain. BS255 is a *virR* deletion derivative of BS183 which contains a previously isolated *inv::lacZ* operon

fusion (Maurelli and Curtiss, 1984). Thus, BS255 produces β -galactosidase constitutively at 37°C and 30°C. A pAEH001 transformant of BS255 was picked and its levels of β -galactosidase at 37°C and 30°C compared to the *virR* deletion mutant BS255 and the original *inv::lacZ* fusion mutant BS183. The transformant expressed reduced levels of β -galactosidase at 30°C which resulted in a 37°C to 30°C β -galactosidase ratio of 15 (Table 8). This result would be expected if the clone harbored a *virR* homolog. The 37°C to 30°C ratio, although not as high as that of the parental fusion mutant BS183, is considerably higher than the *virR* mutant BS255 which had a 37°C to 30°C ratio of <2. Based on the complementation of the *virR* defect and resultant temperature regulation of the *inv::lacZ* operon fusion in BS255, we are able to conclude that the pAEH001 clone contains a gene(s) homologous to *virR*.

VirR⁺ subcloning from *S. flexneri* cosmid clone pATM003. A strategy similar to the one described for cloning the *virR* homolog was used to isolate DNA fragments from *virR* cosmid clone pATM003 in order to identify the smallest DNA sequence which exhibited VirR⁺ activity. The multi-copy number cloning vector pBR329 (Covarrubias and Bolivar, 1982) was used to isolate random restriction fragments from pATM003 and to generate subclones. Due to the inefficiency of transformation in *S. flexneri*, recombinants were first selected and screened in *E. coli* DH5 α . pBR329 clones were selected based on the expression of vector encoded antibiotic resistance genes (Ap^R; Cm^R; Tc^R) and screened by counterselection for antibiotic resistance genes insertionally inactivated by the cloned DNA fragments. Recombinant plasmids were isolated and transformed into *S. flexneri* BS211 and screened

TABLE 8. Regulation of *inv::lacZ* operon fusion in BS255 by GalU⁺ clone^a.

Strain	Relevant characteristics	Units β -galactosidase ^b		Ratio 37°C / 30°C
		37°C	30°C	
BS183	<i>inv::lacZ</i> VirR ⁺	220	5	44
BS255	<i>inv::lacZ</i> VirR ⁻	221	122	1.8
BS255/pCVD301	Cloning vector	270	162	1.6
BS255/pAEH001	GalU ⁺ cosmid clone	47	3	15

^a Values presented are based on representative experiments.

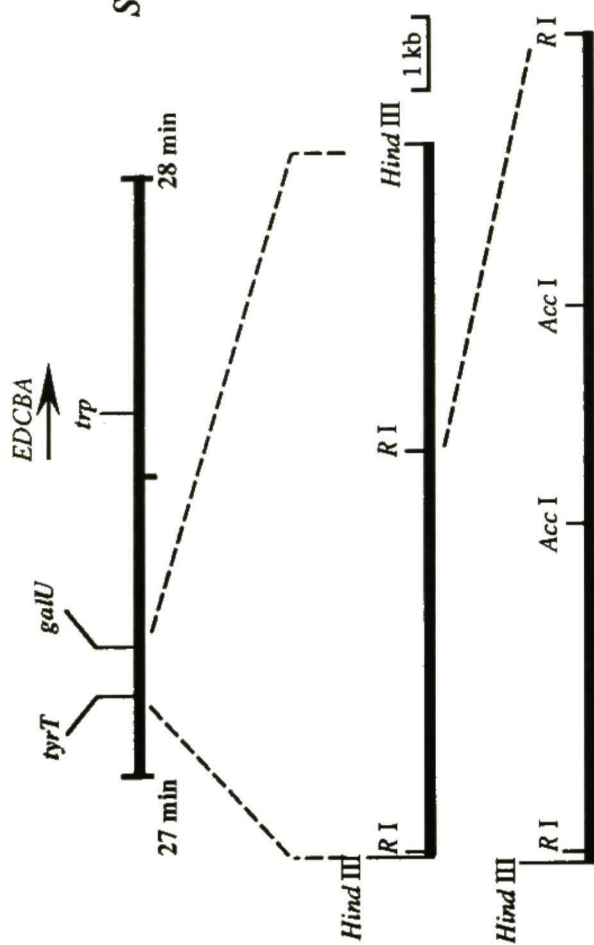
^b Units of β -galactosidase expressed are as defined by Miller (Miller, 1972).

for expression of the VirR^+ phenotype. BS211 is a ΔvirR derivative of the $\text{virR}::\text{Tn10 inv}::\text{lacZ}$ mutant strain BS185 (Table 2). The VirR^- phenotypes expressed by BS185 and BS211 are indistinguishable when levels of β -galactosidase expressed by both strains are measured at 30 and 37°C. VirR^+ activity was defined as the ability to complement the ΔvirR mutation and restore temperature-regulated expression of β -galactosidase as observed for BS211/pATM003 transformants.

Recombinant plasmid pAEH100 was the first subclone identified which complemented the BS211 ΔvirR mutation (Fig. 10). BS211 transformants of pAEH100 or its deletion derivative pAEH102, expressed enzyme levels at both 30 and 37°C which were comparable to the wild-type β -galactosidase levels expressed by BS184 at 30°C (Table 9). The VirR "super" phenotype (VirR^s) expressed by these clones was presumed to be a result of VirR overexpression due to multiple copies of the cloned virR .

Three additional clones which complemented the ΔvirR mutation were pAEH110, pAEH122 and pAEH104. pAEH110 represented a VirR^+ subclone of pATM003. Based on the physical maps and insert fragment orientations, the 5.6 kb *EcoRI* fragments cloned in both pAEH110 and pAEH102 were determined to be identical. The VirR^+ phenotype expressed by pAEH110 suggested that the pAEH100 and pAEH102 VirR^s phenotype was independent of a gene dosage effect. pAEH122, a VirR^+ subclone of pAEH110, contained the right hand 1.8 kb *EcoRI*-*AccI* restriction fragment shown in Figure 10 while pAEH104, a subclone of pAEH102, contained the left hand 2.3 kb *EcoRI*-*AccI* restriction fragment (Fig. 10). As observed for the virR cosmid clone and virR homolog clone, VirR^+ clones pAEH110 and pAEH122 repressed expression

Figure 10. Subclones of VirR⁺ cosmid clone pATM003 and their phenotypic characteristics. (*Top*) Chromosomal region at 27 to 28 minutes on the *S. flexneri* 2a/*E. coli* K-12 physical map. (*Bottom*) Subclones of pATM003. The VirR and GalU phenotypes expressed by the clones are shown on the right. Boxes shown on the 5.6 kb *Eco*RI pAEH110 insert fragment represent the coding sequences of the genes indicated and arrows show direction of transcription.



Shigella / *E. coli* K-12 chromosome

	VirR	GalU
pAEH100	S	+
pAEH102	S	+
pAEH110	+	+
pAEH104	P	-
pAEH103	-	-
pAEH122	+	+

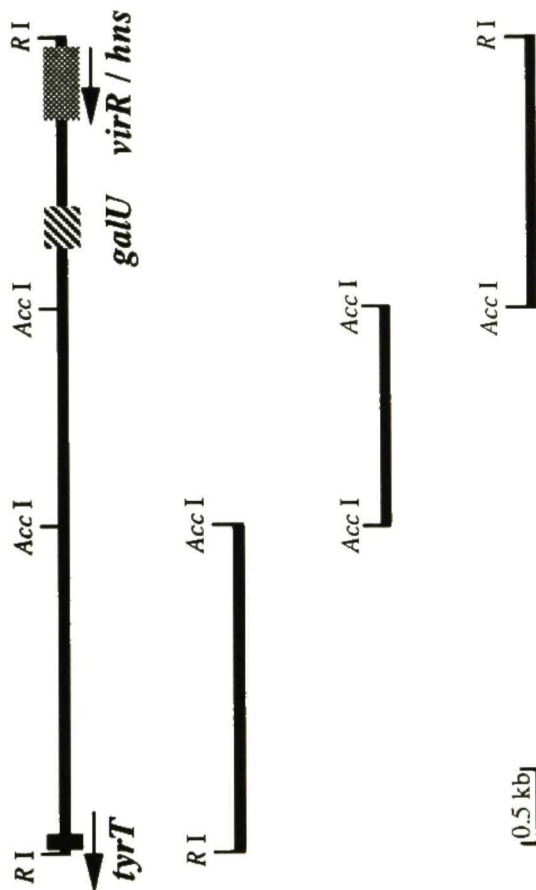


TABLE 9. Complementation of $\Delta virR$ mutation in *inv::lacZ* strain BS211^a.

Strain	Relevant characteristics	Units β -galactosidase ^b		Ratio 37°C / 30°C
		37°C	30°C	
BS184	<i>inv::lacZ</i>	497	11	45
BS211	<i>inv::lacZ</i> $\Delta virR$	507	175	2.9
<u>BS211 + <i>virR</i> clones</u>				
pATM003	GalU ⁺ cosmid clone	342	14	24
pAEH100	VirR ^S	4	9	2.3
pAEH102	VirR ^S	7	12	1.7
pAEH110	VirR ⁺	125	5.4	23
pAEH104	VirR ^P	357	26	14
pAEH122	VirR ⁺	159	6.3	25
pAEH122XBI	<i>virR::XbaI</i> linker mutant	607	211	2.9
pAL13	<i>E. coli pilG</i> clone	109	5.6	19

^a Values presented are based on representative experiments.^b Units of β -galactosidase expressed are as defined by Miller (Miller, 1972)

of β -galactosidase from the reporter gene fusion at both 30 and 37°C (Table 9).

In contrast to the VirR^+ clones, β -galactosidase levels expressed by BS211/pAEH104 at 37°C resembled those expressed by the reporter strain BS211 as opposed to the levels measured for BS211/pATM003 (Table 9). However, β -galactosidase levels expressed by BS211/pAEH104 at 30°C were consistently 3-fold greater than the levels of β -galactosidase measured for BS211 transformants which harbored a VirR^+ clone. pAEH104 therefore, only partially suppressed the ΔvirR mutation and was designated VirR^P (Table 9). Thus, pAEH122 and pAEH104 represented clones of two unique non-overlapping DNA sequences which complemented a ΔvirR mutation and mapped to a 5.6 kb region of the *S. flexneri* chromosome. These complementation studies suggest that the *virR* locus mapped to a region of the *S. flexneri* chromosome defined by this 5.6 kb *EcoRI* fragment.

Mapping *virR*. The identification of two distinct sequences which complement the ΔvirR mutation is somewhat confusing if the mutation represents a deletion of DNA sequences in a single gene. A *virR* gene duplication in the *S. flexneri* chromosome could explain the VirR^P phenotype expressed by pAEH104. Both the 1.8 kb and 2.3 kb *EcoRI*-*AccI* fragments may represent two different copies of *virR*. To test this hypothesis, the VirR clones were used in ΔgalU complementation and Southern hybridization analyses to map the BS185 *virR*::*Tn10*, BS211 ΔvirR mutations and the wild-type *virR*.

As mentioned previously genetic studies indicate that *virR* is located between *galU* and the *trp* operon, closely linked to *galU* on the *S.*

flexneri genetic map (Fig. 10; Maurelli and Sansonetti, 1988). To obtain a first approximation of the location of the VirR complementing clones relative to *galU*, the VirR clones were transformed into *E. coli* ATM016 ($\Delta galU$) and screened for complementation of the $\Delta galU$ mutation. GalU⁺ transformants were identified by their ability to grow in the presence of galactose as a sole carbon source. Transformants which harbored the VirR^s clones pAEH100 and pAEH102 expressed a GalU⁺ phenotype, as did VirR⁺ clones pAEH110 and pAEH122 (Fig. 10). ATM016/pAEH104 transformants however, remained GalU⁻. On the basis of these results, *galU* was shown to map to the 1.8 kb *EcoRI*-*AccI* fragment shown in Figure 10. The localization of *galU* to this DNA sequence and the predicted gene order of *galU-virR-trp* would also indicate that *virR* mapped only to this 1.8 kb *EcoRI*-*AccI* fragment.

Further evidence that *virR* maps to the 1.8 kb *EcoRI*-*AccI* fragment was obtained by Southern blot hybridization. Chromosomal DNA from the $\Delta virR$ strain BS211, wild-type strain 2457T, and *virR::Tn10* strain BS185 was prepared, digested with either *EcoRI* alone or *EcoRI*-*SalI*, and probed with the 5.6 kb *EcoRI* fragment from pAEH110 (Fig. 11). *SalI* recognizes the *AccI* restriction sites in pAEH110, and, therefore, the identical sites should be recognized in the chromosomal digests. As shown in Figure 11, the probe DNA hybridized with a single 5.6 kb fragment in the *EcoRI* chromosomal digests of the wild-type strain (Panel A, lane 2) and also hybridized with 2.3, 1.8, and 1.3 kb fragments in chromosomal DNA preparations from 2457T digested with *EcoRI*-*SalI* (Panel A, lane 3). The molecular weights of these hybridizing fragments correspond in size to the *EcoRI*-*AccI* restriction fragments of the 5.6 kb *EcoRI* fragment of

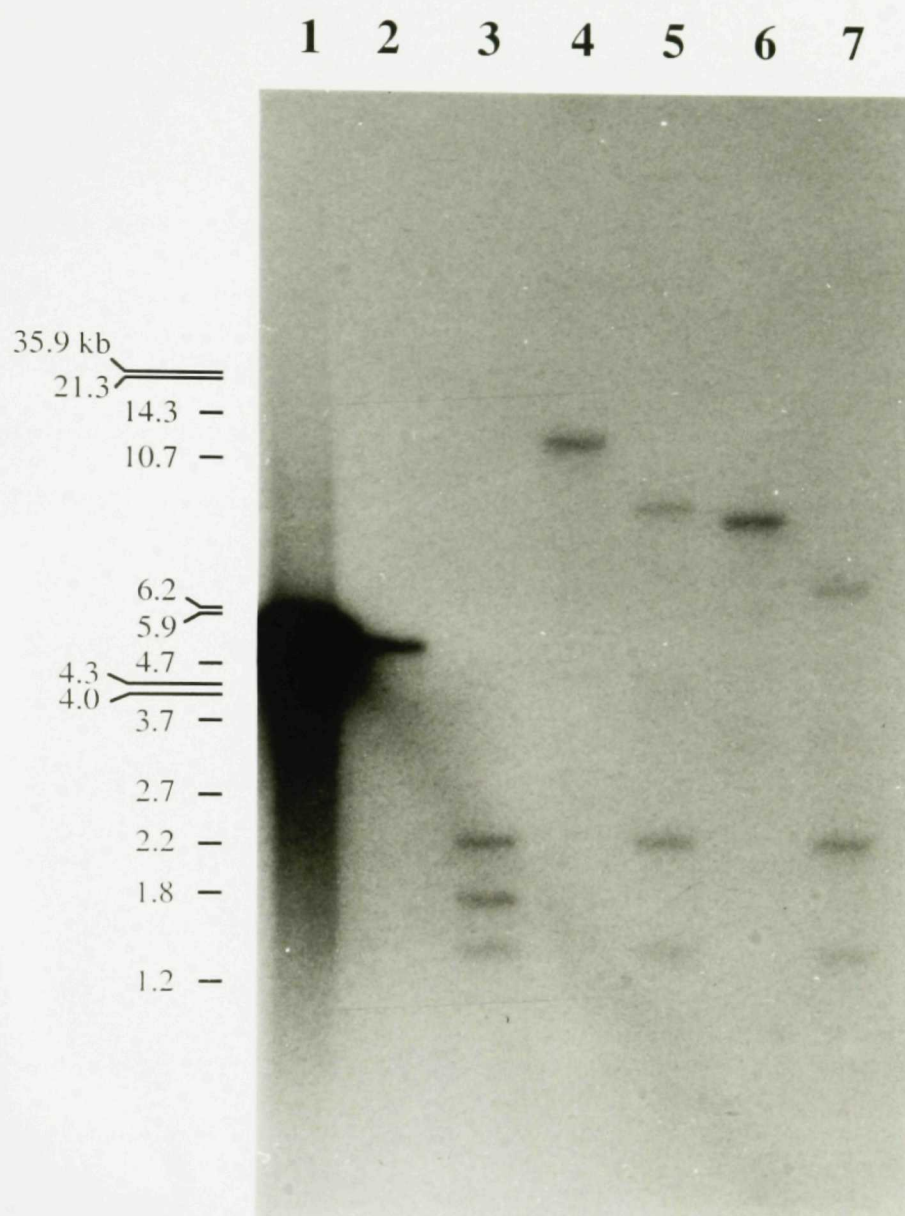


Figure 11. Mapping of *virR* by Southern hybridization analysis. Autoradiogram of Southern blot of wild-type and *virR* mutant strain chromosomal DNA using the 5.6 kb *EcoRI* fragment and as the probe DNA. Lanes 1, probe DNA; 2, 4, 6 *EcoRI* digested and 3, 5, 7 *EcoRI-SalI* digested chromosomal DNA from 2457T (lanes 2 and 3), BS185 (lanes 4 and 5), and BS211 (lanes 6 and 7).

pAEH110 (Fig. 10). In contrast to the wild-type strain, hybridization of the probe DNA with *EcoRI* digested chromosomal DNA from BS185 and BS211 detected single DNA fragments of 12 kb and 8 kb, respectively. *EcoRI* digestion of *Tn10* cleaves the transposon into two fragments of 6.3 and 3.0 kb. Therefore, hybridization of the probe to a single restriction fragment in the BS185 sample predicts that the *Tn10* insertion site is very close to one end of the 5.6 kb *EcoRI* fragment. The 12 kb fragment detected in BS185 probably represents the sum of the 6.3 kb *Tn10* fragment plus the 5.6 kb *EcoRI* fragment. In the *EcoRI-SalI* chromosomal digests of BS185 and BS211, the probe DNA hybridized to three DNA fragments two of which (2.3 and 1.3 kb) were identical in size to those observed for the *EcoRI-SalI* digest of 2457T chromosomal DNA. The third fragment in both BS185 and BS211 was larger than the 1.8 kb fragment seen with the wild-type DNA: 8 kb for the BS185 and 7.5 kb for the BS211 chromosome digests. The 8 kb fragment detected in the BS185 chromosomal DNA corresponds in size to the 6.3 kb *EcoRI Tn10* fragment plus the 1.8 kb *EcoRI-AccI* fragment which indicates that the *Tn10* insertion is located in the 1.8 kb DNA fragment and places its location close to the *EcoRI* site. Furthermore, hybridization of the probe to fragments of 2.3 and 1.3 kb in the BS211 chromosomal digest indicates that the $\Delta virR$ does not extend to the left of the *AccI* site in the 1.8 kb fragment. Based on these Southern hybridization experiments, the *Tn10* insertion and the $\Delta virR$ mutation were localized precisely to a 1.8 kb *EcoRI-AccI* fragment. These results, together with the $VirR^+$ phenotype expressed by pAEH122, confirmed that *virR* maps to this region of the *S. flexneri* chromosome. Moreover, these experiments demonstrated that no significant homology was detected between

the 2.3 kb and 1.8 kb *EcoRI*-*AccI* fragments which suggested that, rather than a *virR* gene duplication, pAEH104 represented an additional genetic locus capable of complementing the $\Delta virR$ mutation.

Homology between *virR* and the *E. coli hns* gene. (i) Comparison of pAEH122 and the *pilG* clone, pAL13. Several independent reports have identified and characterized regulatory mutations which all map to 27 minutes on the *E. coli* chromosome (Higgins *et al.*, 1989; Göransson *et al.*, 1990; Spears *et al.*, 1986; Diderichsen, 1980; Defez and DeFelice, 1981). The phenotypes expressed by strains which carry these different mutations suggest that these loci are allelic. One of the regulatory loci identified in the aforementioned studies is *pilG*, a gene required for the regulation of type 1 piliation in *E. coli* (Spears *et al.*, 1986). Detailed characterization of this locus indicates that *pilG* is equivalent to *hns* (Kawula and Orndorff, 1991), the *E. coli* gene which encodes for a histone-like protein (Pon *et al.*, 1988). Based on the regulatory phenotype associated with *virR* as well as the localization of the *virR* locus to 27 minutes on the *Shigella* genetic map, a *pilG* clone, pAL13, was tested for *virR* complementing activity. pAL13 consists of a 1.8 kb *EcoRI*-*SalI* fragment isolated from a bacteriophage λ clone which represents a segment of the *E. coli* chromosome at 27 minutes (Kohara *et al.*, 1987). BS211/pAL13 transformants expressed a *VirR*⁺ phenotype (Table 9) and pAL13 complemented the ATM016 $\Delta galU$ mutation. Southern hybridization of restriction digests of the *VirR*⁺ clones pAEH110, pAEH122, and the *VirR*^P clone pAEH104 with the 1.8 kb *EcoRI*-*SalI* insert from pAL13 as a probe, demonstrated that this DNA fragment hybridized only with the 1.8 kb *EcoRI*-*AccI* fragments of pAEH110 and

pAEH122 (represented by Fig. 12). In addition, Southern hybridization of the same DNA using an *hns* sequence-specific synthetic oligomer as a probe resulted in an identical hybridization pattern (Fig. 12). Therefore, these observations indicated that the 1.8 kb *EcoRI-SalI* fragment from *E. coli* represented a region very similar to the 1.8 kb *EcoRI-AccI* fragment cloned from *S. flexneri*. These data also presented strong evidence that *virR* and *hns* are alleles.

(ii) Nucleotide sequence analysis of *virR*. Further confirmation that *virR* is allelic to *hns* was obtained by nucleotide sequence analysis of a portion of the 1.8 kb *EcoRI-AccI* fragment in *virR* clone pAEH122. The entire sequence of the region indicated as *hns* in Figure 10 was determined from both strands of pAEH122 using synthetic oligomers to prime the sequencing reactions (Fig. 13). A single large ORF (466 bp) was identified within the sequenced region starting with the ATG at position 93 and ending at position 507. The nucleotide sequence within the ORF was nearly identical to the *E. coli hns* coding sequence with a single base pair change at position 201. Two additional base pair changes were observed outside of the *hns* coding sequence.

(iii) Insertional mutagenesis of the ORF of pAEH122. To confirm that the ORF identified by nucleotide sequence analysis represented the *virR* coding sequence, an *XbaI* linker mutant of pAEH122 (pAEH122XBI) was constructed. The 8 bp *XbaI* linker was inserted into the unique *HpaI* site of pAEH122 which lies within the ORF (Fig. 13). Insertion of the linker should cause a frameshift mutation in the ORF and thereby inactivate the gene product expressed by the ORF. pAEH122XBI was transformed into BS211 and the transformants were assessed for β -galactosidase expression

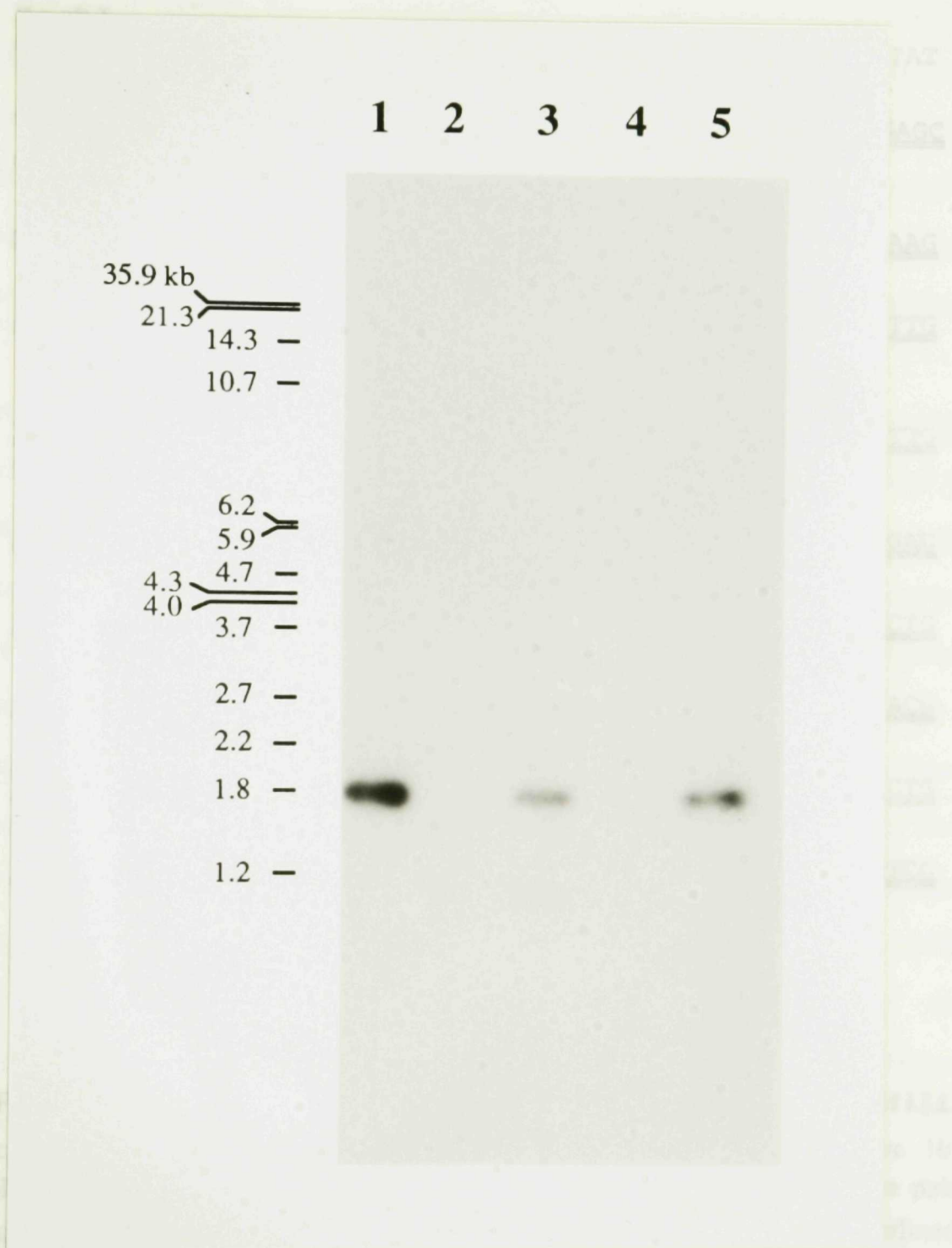


Figure 12. Determination of DNA sequence homology between *hns* and *virR* clone pAEH122 by Southern blot hybridization. Probe DNA used was *hns* sequence specific oligonucleotide. Autoradiogram represents hybridization pattern exhibited by the use of 1.8 kb *EcoRI*-*SalI* insert fragment from *pilG* clone pAL13 or *hns* sequence specific oligonucleotide as probes. Lanes: 1, *EcoRI*-*SalI* digested pAL13; 2, *EcoRI*-*AccI* digested pAEH104; 3, *AccI* digested pAEH122; 4, *EcoRI*-*AccI* digested pAEH103; 5, *EcoRI*-*AccI* digested pAEH110.

EcoRI

1 GAATTCCTTA CATTCCTGGC TATTGCACAA CTGAATTTAA GGCTCATTAT
 51 TACCTCAACA AACCCAGCCC CAATATAAGT TTGAGATTAC TACA**ATGAGC**
 C C
 101 GAAGCACTTA AAATTCTGAA CAACATCCGT ACTCTTCGTG CGCAGGCAAG
 151 AGAATGTACA CTTGAAACGC TGGAAGAAAT GCTGGAAAAA TTAGAAGTTG

HpaI

201 TTGTTAACGA ACGTCGCGCG AAGAAGAAAG CGCGGCTGCT GCTGAAGTTG
 C
 251 AAGAGCGCAC TCGTAAGCTG CAGCAATATC GCGAAATGCT GATCGCTGAC
 301 GGTATTGACC CGAACGAACT GCTGAATAGC CTTGCTGCCG GTTAAATCTG
 351 GCACCAAAGC TAAACGTCTC AGCGTCCGGC AAAATATAGC TACGTTGACG
 401 AAAACGTGCG AAACATAAAC CTGGACTGGC CAAGGCCGTA TCTCCAGCTG
 451 TAATCAAAAA AGCAATGGAT TGAGCAAGGT AAATCCCTCG ACGTATTTCC
 501 TGATCAAGCA **TAA**GCTTTT GTAGATTGCA CTTGCTTAAA AT

Figure 13. Partial nucleotide sequence of VirR⁺ clone pAEH122.

The nucleotides are numbered starting with the *EcoRI* site shown in Figure 10. Nucleotides which are shown beneath the numbered nucleotides represent base pair differences between the *S. flexneri* 2a and *E. coli* K-12 sequences. Double underlined bases represent the 412 bp ORF detected by computer sequence analysis with the bold face letters representing the start and stop codons respectively.

at 30 and 37°C (Table 9). BS211/pAEH122XBI transformants exhibited a deregulated β -galactosidase phenotype and produced enzyme levels similar to those of the BS211 (Table 9). These data, therefore, unambiguously demonstrate that the VirR^+ activity expressed by the 1.8 kb *EcoRI*-*AccI* fragment cloned in pAEH122 was due to expression of the ORF identified by nucleotide sequence analysis and establish that *virR* is an allele of *hns*.

Characterization of the VirR^P clone. (i) **Specificity of VirR^P activity.** The criteria used to define *virR* activity in these studies are based on the ability of clones to complement the ΔvirR mutation and restore temperature-regulated expression of a specific reporter gene *lacZ* fusion. It is possible therefore, that the VirR^P activity expressed by pAEH104 may be specific only to this particular operon fusion and not reflect a general regulatory effect on *Shigella* virulence gene expression. Thus, the regulatory effects of pAEH104 on the expression of a different *lacZ* operon fusion and the Ipa proteins were examined. pAEH104 was transformed into the *virR::Tn10* derivative of the *mxi::lacZ* 11.5 fusion strain BS226 (named BS303) and β -galactosidase levels were measured for the transformants at both 30 and 37°C (Table 10). BS303/pAEH104 exhibited reduced levels of β -galactosidase expression at both temperatures when compared to the parental *virR::Tn10* strain. At 37°C the levels of β -galactosidase expressed by the pAEH104 transformant were comparable to the wild-type *virR mxi::lacZ* fusion strain whereas at 30°C the levels of enzyme expressed were approximately 5-fold higher than the parental fusion strain (Table 10). These results resembled those observed for BS211/pAEH104 (Table 9) since the enzyme levels at 30°C for BS303/pAEH104

TABLE 10. Complementation of *virR::Tn10* mutation in *mxi::lacZ* strain BS303 by pAEH104^a.

Strain	Relevant characteristics	Units β -galactosidase ^b		Ratio 37°C / 30°C
		37°C	30°C	
BS226	<i>mxi::lacZ</i>	638	14	46
BS303	<i>mxi::lacZ virR::Tn10</i>	1104	193	5.7
BS303 /pAEH104	VirR ^P clone	608	65	9.3

^a Values presented are based on representative experiments.

^b Units of β -galactosidase expressed are as defined by Miller (Miller, 1972)

represented only a partial repression of β -galactosidase expression at this temperature. It was concluded from these results that the VirR^P phenotype expressed by the 2.3 kb *EcoRI*-*AccI* fragment was not restricted to a particular reporter gene. To rule out the possibility that the VirR^P phenotype reflects a regulatory mechanism particular to the expression of the *lacZ* portion of the operon fusions tested, the effects of pAEH104 on Ipa protein expression was examined. Western blot analysis of whole cell bacterial lysates of BS185/pAEH104 grown at 30 or 37°C revealed that the Ipa protein profile observed for this strain was identical to that of either the wild-type strain 2457T or wild-type *virR inv::lacZ* fusion strain BS184 (Fig 14). Ipa protein expression in BS185/pAEH104 lysates prepared from bacteria grown at 30°C and 37°C appeared to express similar levels of protein as the wild-type *virR* strains. Thus it was concluded that the VirR^P phenotype expressed by pAEH104 was not an artifact of the *inv::lacZ* operon fusion harbored in BS211.

(ii) Nucleotide sequence analysis of the pAEH104. The 2268 bp nucleotide sequence of the pAEH104 *EcoRI*-*AccI* fragment was determined by the strategy shown in Figure 15. The sequence of one strand was obtained by synthetic oligomer priming of the double stranded pAEH104 template at various sites along the sequence. The opposite strand was sequenced from a series of nested deletion derivatives of the 2.3 kb fragment using the M13 -40 synthetic oligonucleotide primer. To facilitate the isolation of Exonuclease III generated nested deletions, recombinant plasmid pAEH106 was constructed. pAEH106 represents the 2.3 kb *EcoRI*-*AccI* fragment from pAEH104 modified by the addition of *XbaI* oligonucleotide linkers and

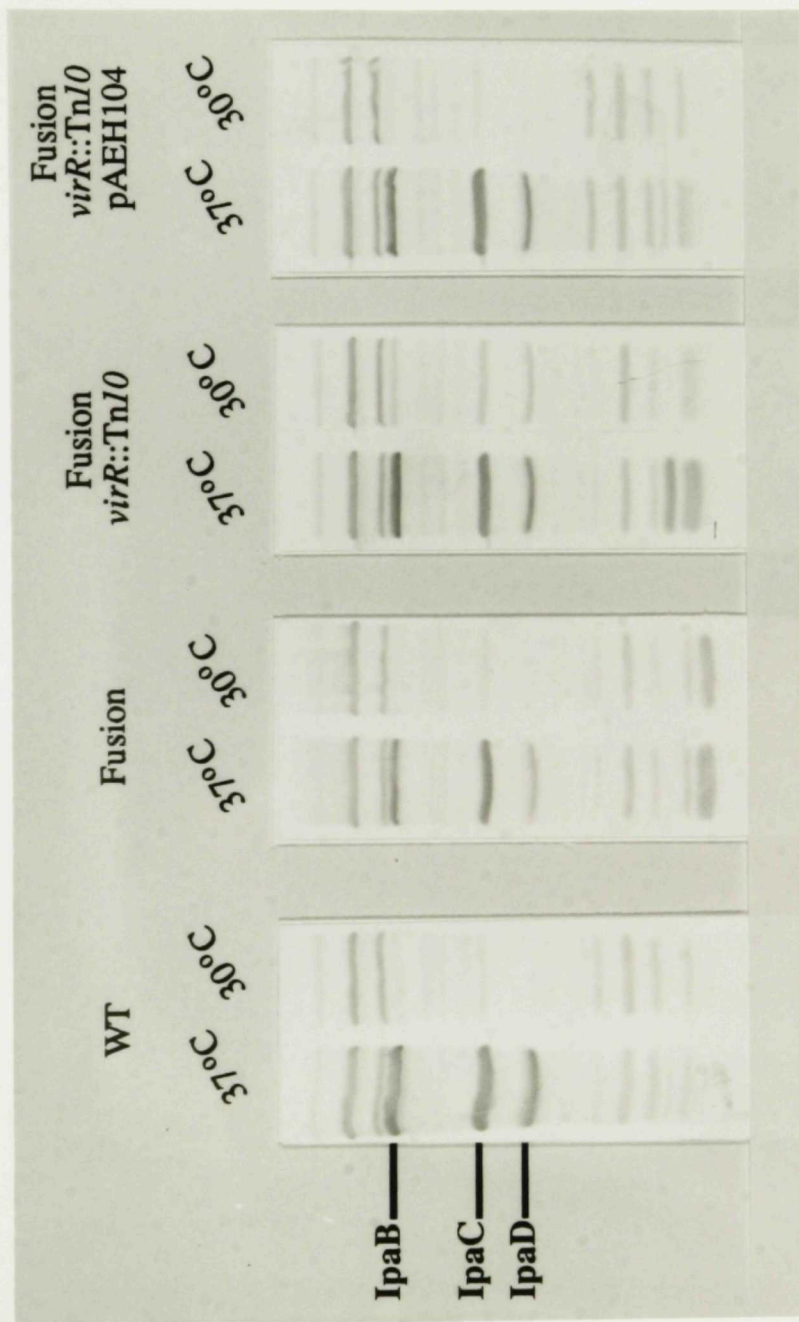


Figure 14. Effect of *VirR^P* activity on Ipa expression. Western blot of whole-cell bacterial lysates from strains cultured at 37°C and 30°C and probed with convalescent-phase human polyclonal antiserum (1:100 dilution) from a patient infected with *S. flexneri* 2a. Lanes: WT, 2457T; Fusion, BS184; Fusion *virR::Tn10*, BS185; Fusion *virR::Tn10* pAEH104, BS185 transformant harboring *VirR^P* clone pAEH104.

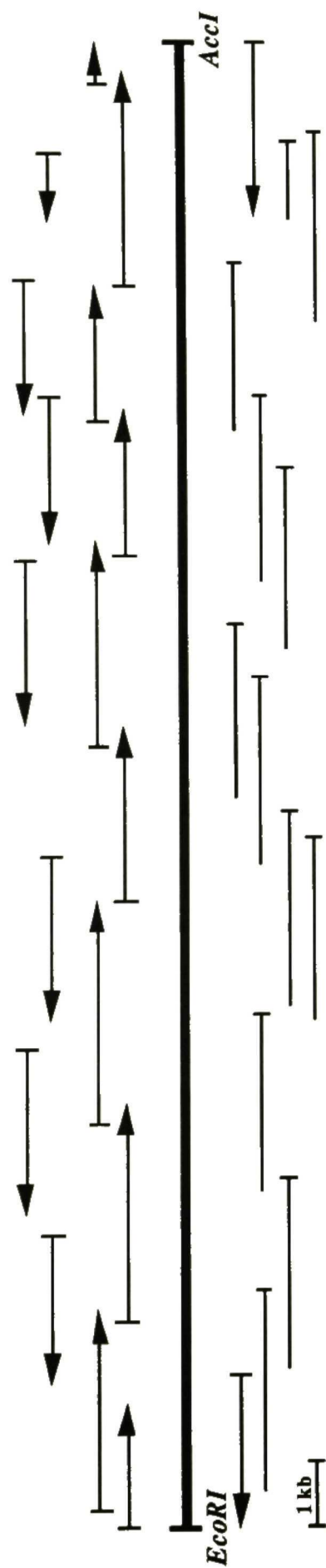


Figure 15. Sequencing strategy for the 2.3 kb *EcoRI*-*AccI* fragment of pAEH104 and pAEH106. The thick line represents the 2.3 kb *EcoRI*-*AccI* fragment cloned into pBR329 (pAEH104) and pUC19 (pAEH106). Arrows indicate the direction and the length of sequence determined by oligonucleotide primed sequencing reactions. The thin lines below the cloned insert DNA represent the nested deletions generated in recombinant plasmid pAEH106, with vertical lines indicating the endpoint of the deletions. The length of each line reflects the number of nucleotides sequenced from each nested deletion clone.

cloned into the *Xba*I restriction site of the *E. coli* cloning vector pUC19 (Yanisch-Perron *et al.*, 1985).

The complete nucleotide sequence of the *Eco*RI-*Acc*I fragment (Fig. 16) was examined for related sequences in the GenBank DNA Sequence Data Base using the GCG Sequence Analysis Package Word Search program (Devereux *et al.*, 1984). From the guanosine in the *Eco*RI site at position 1 (Fig. 16) to the cytosine at position 676, a high degree of nucleotide sequence identity was seen with the reported sequence of the *E. coli tyrT* locus (Goodman *et al.*, 1968). Detailed comparison of the related sequences using the GCG Bestfit program, revealed that two regions of 99% overall sequence identity existed between the pAEH104 insert and *tyrT* (Fig. 17). Nucleotides 1-24 and 25-676 of the *Eco*RI-*Acc*I fragment were aligned with two segments of the *tyrT* sequence, separated by 630 nucleotides. These sequence analyses indicate that the *S. flexneri tyrT* locus derived from 2457T is naturally deleted for 630 bp of sequence found in the *E. coli tyrT* locus. The Southern hybridization results presented in Figure 11 (Panel B) substantiate the presence of the *Shigella tyrT* deletion since the 2.3 kb *Eco*RI-*Sal*I restriction fragment was detected in the wild-type *S. flexneri* chromosome. Therefore, the sequence differences seen between the *S. flexneri* and *E. coli* K-12 *tyrT* loci were not a subcloning artifact.

Curiously, the pAEH106 clone was unable to complement the $\Delta virR$ mutation when transformed into BS211. Transformants expressed levels of β -galactosidase indistinguishable from those expressed by BS211. This observation suggested that the lack of *VirR* activity may be the result of

EcoRI **AvaI**

1 GAATTCGGG GCTTTTGAAA GTGATGGTGG TGGGGGAAGG ATTGGAACCT TCGAAGTCGA TGACGGCAGA TTTACAGTCT GCTCCCTTTG GCCGCTCGGG

101 AACCCACCA CGGGTAATG CTTTTACTA GCCTGCTCC TTATCGGGAA GCGGGGCGCA TCATATCAAA TGACGCGCCG CTGTAAAGTG TTACGTTGAG

201 AAAAATGAAC TGGTTGCGTA ATTTTCATCC GTAACGGATT AAAGTAACC AGTTATTTT GCTGGCGATT AAAGAATAAT CGTCCGATTA CCGTAAACAA

AatII

301 AGACGCGTTG CGCCAGTACT TTGTATAGCG CACGACTTAA GACGTTTTT TCGACGTCAC GACCTGCGCG CATCATATCT TCAGCTGTGT AGGTATGATC

401 GACATGAATA ACGTCCTGCA TGATGATTGG GCCTCGTCC AGATTGTCAT TCACATAGTG AGCGGTTGGG CCAATAATCT TCACACCACG TTCATAGGCC

501 TGGTGATAAG GACGTGCGCC GATAAACGCA GGCAGGAATG AATGGTGAAT ATTGATGATT TTATTCGGGA AGCGTGCCAC AAATTCGGCG GTTAATACCC

601 GCATATACTT CGCCAGCACC ACATAGTCAG GTGATAAGC ATCAATGGCG TCGCGCATCT TTGATCGTG CTCGTTGCGG CTTAACCCCT CATGGCTTAC

701 CAGCTCAAAC GGAATATCAA AACGCTCAAC CAGAGAACGT AAAGTATCGT GGTTACCAAT AACTGCCGCG ATTTGACAT CCAGACCGCC GTAATTGGCT

801 TTCATCAACA AATCGCCAAG GCAATGCGCT TCTTTAGTGA CCAGAATCAC TATCCGGCGA CGACCGGCAG GATTAGCTC ACGCAGGAG CCTTCTGGCA

BclI

901 ATGCGCTATC GAGATCCGCC AGCAGGGTGG AATCATIAAA AATCCCTTCC AGTTCGGTGC GCATAAAAAA GCGCCCGGTA CGGTGATCAA CAAATTCATT

1001 GTTCTGTACG ATATTAACT CGTGCTTGTA GCAAATATTG GTAATACGTG CGATCAGACC TTTTGGTCC GGACAAATAG TACGCAGAAC TTTACGTTGG

DraI

1101 AGTGAATGCA TTGCTGGAAA AACCTTGTG AGAGTGTGTT CTAACCGTA CCGTCAACCA TTATTGGCCG CAGCACTTTT TAAATTTTTT ACCTGAACCA

1201 CAAGGGCAGG GATCGTTGCG ACCAACTGC GGACGTGTAC CGTCAATATA GTACCATGA CCGTTTCTT TCAAAAATCG CGAACGTTCA ATTATTGCAC

1301 CTGTTTTGCC ACCTTCGGTA AATCGAGCAA CAAAAGTAC AAATCCGATA TTGTCGGCGT CCTGCCAACA ATGTTCAAAT ACGGTCAATC CCAACCATTC

1401 AGTATGCGCA AATCCGGTCA TCAATTCGGC ACGTAACGGC CGCTGTCCAC AAGAGGGATG CCAGGTCTTT ATTAATAAT CAGCGTCTTG CATCACAAAA

1501 GCGCAGTAAC GCGAACCGAT GAGATGTTCT GGATCAGGTG CAACCTTTT ACCAGACACA TAAGGGTGGC AACATAGGCT ATACTCGACA GCACTACCAC

1601 AGGGACAAAG CTGAGACACA AATAATCTCC CTGGAACAA TAACGGCGTA TTAACCGCCT GAGTAGCACT ATGTTAACCG AGCAGTAGCG ATGTGGCTAC

1701 GATTGCATTC CAGGGGAATC TTGCGGGAAT AATGAGAAAG ATAAAAATAG GGCTGGCGCT GGGATCTGGC GCGGCGAGAG GTTGGTCGCA TATTGGCGTT

EcoRV

1801 ATTAATCCGC TAAAAAAGT GGGTATTGAA ATTGATATCG TTGCAGGATG TTCAATTGGT TCGCTGGTGG GCGCGGCCTA TGCATGCGAT CGATTATCTG

1901 CGCTGGAAGA TTGGGTAACC TCTTTCAGTT ATTGGGAGCT TTACGCCTG ATGGACCTCT CCTGGCAGCG CGGTGGGTTA CTGCGGGGCG AGCGTGTCTT

2001 CAATCAATAT CGCGAAATAA TGCCGGAAAC AGAGATCGAA AATTGTTCCT GTGCTTTGC GCCTGTTGCC ACCAATTTAA GTACGGGGCG TGAATTATGG

2101 TTTACTGAAG GCGATCTCCA TCTTGCTATT CGCGCATCAT GCAGTATTC AGGACTCATG GCACCGGTTG CACATAACGG CTACTGGCTG GTTGATGGGG

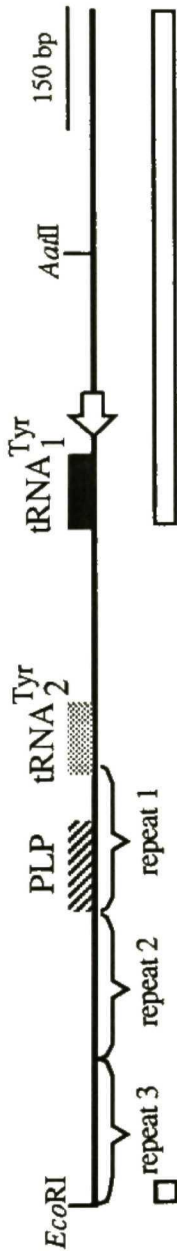
AccI

2201 CAGTAGTTAA CCCAATTCCT ATTTCTCTTA CGCGTGCAAT GGGGGCTGAT ATTGTGATAG CGGTCGAC

Figure 16. Nucleotide sequence of the 2.3 kb *EcoRI*-*AccI* fragment from *VirR^P* clone pAEH104. The nucleotides are numbered starting with the *EcoRI* site shown in Figure 10. Nucleotides which are underlined represent the *S. flexneri* 2a sequence which exhibited sequence identity to that of the *E. coli* *tyrT* locus (described in further detail in Fig. 17).

Figure 17. Nucleotide sequence comparison of *S. flexneri* 2a and *E. coli* K-12 *tyrT* loci. (Top) Organization of *E. coli tyrT* locus: black box represents coding sequence of tRNA^{Tyr} copy 1; stippled box represents coding sequence of tRNA^{Tyr} copy 2; cross hatched box (PLP) represents coding sequence of protamine-like protein; repeats 1-3 are contiguous 178 bp identical repeats; open arrow represents location of tRNA^{Tyr} promoter sequence; Restriction sites for *EcoRI* and *AatII* are identical to those shown in Figure 16; open boxes represent the *S. flexneri* serotype 2a *tyrT* locus. (Bottom) Alignment of nucleotide sequences showing sequence identity from of two *tyrT* loci. SF and EC denote the *S. flexneri* and *E. coli* K-12 sequences, respectively. Vertical bars denote bases which are identical. Absence of a vertical bar indicates a base pair mismatch between the two sequences. Nucleotides which are double underlined represent the extent of the coding sequences of the genes written below the sequences.

E. coli K-12 *tyrT* locus



SF 1 GAATTCGGGGCTTTTGAAGTG 23
 |||||
 EC 1316 GAATTCGGGGCTTTTGAAGTGATGTTGGTGGGGAAGGATTACTCAGCGTGGCGCTTCGCCCTTCGGGTGCTGCCTCGCTGACGCTCGAGCGGAACCTTAGTCGAAGCTTCTCAT
 EC 1180 CCTTCCCGCATGGCGAGATAATTGATTGCGGATTTCGCTTCGAGACTTCAGGACATTTTGAAAGTGATGGTGGGGAAGGATTACTCAGCGTGGCGCTTCGCCCTTCGGCTCGTTCGGCAACGCT
 P_{LP}
 EC 1045 CTCTCGCTGGCGCTCGAGTCGAACCTTGGTCGAAGCTTCTCATCTTCGCCGATGGCGAGAAATATTTAATTGGGATTCGTTGGGAAGTTCAGGACTTTTGAAGTCATGGTGGTGGGGAAGGATTTCGAACC
 rRNA^{Tyr} copy 2
 EC 910 TTCGAAGTCGATCAGCGCAGATTTACAGTCTGCTCCCTTGGCGGCTCGGAAACCCACAGGGGTAAATTCAAATTTTCAGGTAATGCTTGAGATGGTGGTGGGGAAGGATTATTCGCTCGCTCGCTCCTCACC
 rRNA^{Tyr} copy 2
 EC 775 TTTCGGCCGTTGCTGTGGCAACGCTCTCTCGCTTCGATCGAATCGAACCTTAGTCGAAGGTTCTCACCTTCCCGATGAGTGCAAACTTTCACAATCTCACGAAGTTACCAATACGCGCTGTGGTAATTTAT
 ||
 SF GGTGGTGGGGAAGGATTCGAACCTTCGAAGTCGATCAGCGCAGATTTACAGTCTGCTCCCTTGGCGGCTCGGAAACCCACAGGGGTAAATGCTTTTACTAGCTGCTCCCTTACTCGGAAAGCGGGCGCA 160
 |||||
 EC 640 GGTGGTGGGGAAGGATTCGAACCTTCGAAGTCGATCAGCGCAGATTTACAGTCTGCTCCCTTGGCGGCTCGGAAACCCACAGGGGTAAATGCTTTTACTGGGCTGCTCCCTTATCGGGAAGCGGGCGCA
 rRNA^{Tyr} copy 1
 SF ATCATATCAAAATCAGCGCCGCTGTAAAGTGTAACTGGTTCGGTAATTTTCATCCGTACGGAATTAAGGTAACCACTTATTTTCTGGCGGATTAAGAATAATTCGTCGGATTACCGT 295
 |||||
 EC 505 ATCATATCAAAATCAGCGCCGCTGTAAAGTGTAACTGGTTCGGTAATTTTCATCCGTACGGAATTAAGGTAACCACTTATTTTCTGGTGGCGATTAAGAATAATTCGTCGGATTACCGT
 - 10 - 35
 SF ACAAAGACGGTTGCGCAGTACTTTGTATAGCGCAGACTTAAAGACGTTTTCGACGTCAGCACTGGCGGCATCATATCTTCAGCTGTAGGTATGATCGACATGAATAACGCTCTGCATGATGATGG 430
 |||||
 EC 370 ACAAAGACGGCTGTGCGAGTACTTTGTATAGTCACGACTTAAAGACGTTTTCGACGTCAGCACTGGCGGCATCATATCTTCAGCTGTAGGTATGATCGACATGAATAACGCTCTGCATGATGATGG
 GCCTTCGTCAGATTGTCATTCACATAGTACGGGTTGGGCCAATATCTTCACACCAGCTTCATAGGCTGGTTCATAGGACGTCGCGCAATTAACGCTGGCAGGAATGAATGGTGAATATTGATGATCTTATT 567
 |||||
 EC 235 GCCTTCGTCAGATTGTCATTCACATAGTACGGGTTGGGCCAATATCTTCACACCAGCTTCATAGGCTGGTTCATAGGACGTCGCGCAATTAACGCTGGCAGGAATGAATGGTGAATATTGATGATCTTATT
 CGGGAAGCGTGCCACAAATTCGGGCTTAATACCGGCATATATCTTCGCCAGCACCATGTCAGTTGATAAGCATCAATGGCTCCGCCATCTTTTGATC 667
 |||||
 SF CGGGAAGCGTGCCACAAATTCGGGCTTAATACCGGCATATATCTTCGCCAGCACCATGTCAGTTGATAAGCATCAATGGCATCCGCCATCTTTTGATC 1

convergent transcription from the *lac* operon promoter (P_{lac}) on the vector and the gene(s) which complement the $\Delta virR$ mutation. pUC18 (Yanisch-Perron et al., 1985) and pUC19, plasmids which differ only in the direction of their multiple cloning sites, were used to clone the 2.3 kb *EcoRI*-*AccI* fragment from pAEH104 to determine if expression of the $VirR^P$ phenotype was orientation dependent. BS211 transformants of pAEH112 (pUC18 clone), which represents the same fragment orientation determined for pAEH106, expressed a $VirR^-$ phenotype, whereas transformants of pAEH113 (pUC19 clone) expressed a $VirR^P$ phenotype (data not shown). These results suggest that transcription of the gene(s) required for expression of the $VirR^P$ phenotype is in the direction toward the *EcoRI* site at position 1 (Fig. 17) and indicate that the expression of these gene(s) was suppressed in pAEH106 due to convergent transcription with the P_{lac} .

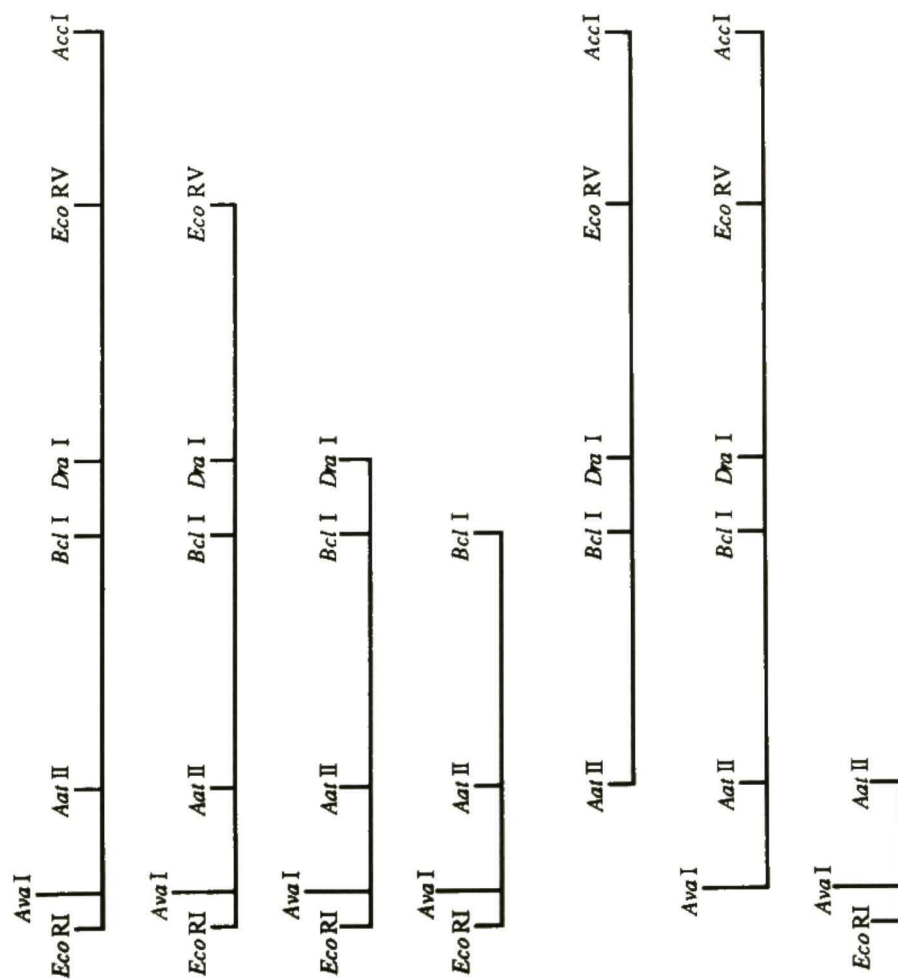
(iii) Mapping of $VirR^P$ activity. A series of recombinant plasmids derived from pAEH104 were used to map the location of the gene(s) encoding $VirR^P$ activity (Fig. 18). The unique restriction sites which denote the endpoints of the various subclones were identified from the nucleotide sequence of the *EcoRI*-*AccI* fragment (Fig. 16), and each subclone represents a fragment cloned into the *EcoRI* and *SalI* sites of pBR329. Incompatible restriction sites were modified by the addition of either *SalI* or *EcoRI* oligonucleotide linkers to permit cloning into these sites. The orientation of the subcloned fragments, therefore, is identical to that of the *EcoRI*-*AccI* fragment of pAEH104. As in previous experiments, BS211 served as the reporter strain into which the pAEH104 subclones were transformed and screened for the ability to confer a $VirR^P$ phenotype.

Figure 18. Mapping of VirR^P activity. Partial restriction maps of VirR^P clone pAEH104 and its subclones are shown on left. The levels of β -galactosidase expressed by BS211/subclone transformants are shown in right hand column. Units of β -galactosidase expressed by the BS211 transformants are as defined by Miller (Miller, 1972) and are based on representative experiments. All of the pAEH104 subclones represent fragments cloned into the *EcoRI*-*SalI* site of pBR329. pAEH114, pAEH116, pAEH117, and pAEH123 represent subclones constructed by the addition of *SalI* oligonucleotide linkers to the *EcoRV*, *DraI*, *BclI*, and *AatII* restriction sites, respectively. pAEH119 and pAEH121 represent subclones constructed by the addition of *EcoRI* oligonucleotide linkers to the *AatII* and *AvaI* sites, respectively.

Units of β -galactosidase

pAEH104 subclones

37°C 30°C



BS211 transformants of pAEH114, pAEH116, pAEH117, and pAEH123 each expressed β -galactosidase levels similar to that of a BS211/pAEH104 transformant at both 30 and 37°C (Fig. 18). These data indicate that over 80% of the *EcoRI*-*AccI* sequence could be deleted and *VirR^P* activity was retained. In contrast, the levels of β -galactosidase produced by BS211 transformants of either pAEH119 or pAEH121 were unchanged from the levels detected for BS211. This indicated that a deletion of only 84 nucleotides from the *EcoRI* site (represented by pAEH121) resulted in a complete loss of *VirR^P* activity. Both subclones pAEH119 and pAEH121 represent either a complete or partial deletion of the tyrosine transfer RNA (*tRNA^{Tyr}*) while pAEH123 is the smallest clone which contains a complete *tRNA^{Tyr}* coding sequence (Fig. 18). The complementation phenotypes expressed by these clones indicate that *tRNA^{Tyr}*, expressed in high copy, is capable of partial complementation of a deletion in the *virR* locus. These results appear to be further substantiated by the loss of *VirR^P* activity in *tRNA^{Tyr}* clones pAEH106 and pAEH112. Based on the nucleotide sequence of the 2.3 kb *EcoRI*-*AccI* fragment, transcription of the *tRNA^{Tyr}* promoter is in the direction of the *EcoRI* site and therefore convergent with the *P_{lac}* promoter in these *tRNA^{Tyr}* clones.

DISCUSSION

Part I:

Characterization of *Shigella* spp. virulence and its regulation has provided evidence that virulence is dependent on the coordinate expression of chromosomal and plasmid-encoded genes (Sansonetti *et al.*, 1981; Sansonetti *et al.*, 1983; Maurelli and Sansonetti, 1988) which results in a temperature-regulated virulence phenotype (Maurelli *et al.*, 1984). Genes which code for different virulence factors have been identified and cloned, yet the total number of virulence genes and the number of virulence genes which are temperature-regulated are not known. In these studies, seven temperature-regulated operon fusions were identified in *S. flexneri* by using the λ placMu *lacZ* operon fusion system (Bremer *et al.*, 1985). The possible roles that these genes play in the virulence phenotype of the organism were examined. It was determined that the seven insertion mutants identified plasmid-encoded, temperature-regulated genes which could be categorized into three distinct classes: 1) virulence genes involved in the invasion step which are regulated by *virR*; 2) non-virulence associated genes which are also under the control of *virR*; and 3) non-virulence associated genes which are independent of *virR* control.

Class 1 mutants: *virR*-regulated *inv* genes.

Four of the temperature-regulated fusion mutants isolated were shown to harbor *lacZ* operon fusions to genes involved in expression of the *Inv*⁺ phenotype. In addition to their inability to invade HeLa cells, assay of

these fusion mutants for the virulence-associated phenotypes of Congo red binding and contact hemolysis confirmed previous observations of a strong correlation between the *Inv*⁻ phenotype and the *Pcr*⁻ and *Hly*⁻ phenotypes (Maurelli *et al.*, 1984; Sansonetti *et al.*, 1986; Clerc *et al.*, 1986). The *Pcr*⁻/*Hly*⁻ phenotypes expressed by the *Inv*⁻ fusions 11.5, 17.7 and 18.15 suggest that the genes inactivated by these operon fusions are also involved in the ability of *Shigella* to bind Congo red and express contact hemolytic activity. Western blot analysis of these non-invasive mutants demonstrated that intracellular expression of the Ipa proteins was unaltered, whereas a whole cell ELISA, used to detect surface IpaB and IpaC expression, indicated an altered recognition of both proteins by their respective MAbs. On the basis of these latter observations, fusions 11.5, 17.7, and 18.15 appeared to be located in genes essential for membrane expression of the Ipa proteins (*mxi*). Mapping of the fusion insertions and subcloning of the *mxi::lacZ* 11.5 fusion promoter indicated that at least two distinct *mxi* genes exist. The *mxi::lacZ* mutant phenotype suggests that the *mxi* genes could represent genes involved in either post-translational modification of the Ipa proteins, or transport and positioning of the Ipa proteins in the outer membrane of the bacteria. Another interpretation of the ELISA data is that the Ipa proteins are surface expressed but masked in such a fashion that they are not recognized by the MAbs. Analysis of membrane fractions indicates that the Ipa proteins are not found in outer membrane fractions of *mxi* mutant strains as they are for wild-type *S. flexneri*, but accumulate in the inner membrane (Andrews *et al.*, 1991). These observations favor a role for the

mxl gene products as factors required for transport of the Ipa proteins to the bacterial outer membrane. Moreover, the *Pcr*⁻ and *Hly*⁻ phenotypes expressed by these fusion strains suggest that the *mxl* genes may serve a more general purpose in the transport of other surface-exposed virulence-associated proteins in *Shigella* spp.

Fusion 17.6 *ipaB::lacZ*⁺, also characterized as a non-invasive fusion mutation, resulted in the synthesis of a truncated IpaB protein and caused a polar mutation which prevented expression of the other *ipa* genes. The nucleotide sequence of *ipaB* and *ipaC* shows that *ipaB* is the first gene in the operon (Baudry *et al.*, 1987; Venkatesan *et al.*, 1988). The polar effect of the *ipaB::lacZ*⁺ 17.6 mutation confirms the previously reported operon nature of the *ipa* locus. The avirulent phenotype expressed by fusion mutant 17.6 also confirms the involvement of the *ipa* genes in invasion, but a specific *ipa* gene cannot be implicated due to the polar nature of the insertion. In addition, the *Pcr*⁺ phenotype expressed by this strain suggests that the Ipa proteins are not involved in Congo red binding while the inability of this strain to lyse SRBC indicates that contact hemolytic activity may require Ipa expression.

Class 2 mutants: *virR*-regulated non-virulence genes.

In contrast to the *Inv*⁻ fusions isolated, three of the seven temperature-regulated *lacZ* operon fusions were still invasive for HeLa cells. Further characterization of these strains in the plaque, Séreny, Congo red binding, and contact hemolytic activity assays indicated that they expressed phenotypic characteristics identical to the parental wild-

type strain 2457T. These results suggest that the genes inactivated by the fusions, although regulated by growth temperature, do not encode products involved in eliciting the virulence phenotype. One must consider, however, the possibility that within the scope of the assay systems used, a mutation in another virulence factor(s) may be undetectable. Another possible explanation for the full virulence phenotype of these operon fusion mutants is that the gene product which results from the *lacZ* insertion is not altered sufficiently to inactivate it. Such an explanation could account for the full virulence phenotype observed in these strains in the presence of temperature-regulated β -galactosidase expression. Therefore, these genes may still fall into the class of *virR*-regulated virulence genes (class 1). Further characterization of these mutants in virulence assay systems such as the ligated ileal loop (Formal *et al.*, 1968) or the Rhesus monkey may help to confirm this possibility.

Class 3 mutants: Non-virulence genes independent of *virR* control. The third class of insertion mutants which were isolated suggests the existence of another *virR*-like repressor. Fusion mutant 18.11 showed no change in β -galactosidase expression after the introduction of a *virR::Tn10* mutation. Other studies have demonstrated the presence of regulatory genes other than *virR* in the expression of *Shigella* virulence, yet these genes have all been identified as activators (Sakai *et al.*, 1988; Adler *et al.*, 1989; Buysse *et al.*, 1990; Watanabe *et al.*, 1990). To provide further evidence that an additional *virR*-like regulatory locus exists in *S. flexneri*, mutations which result in deregulated expression

of β -galactosidase in the 18.15 fusion mutant background would need to be isolated.

Defining the *virR* regulon. Although the 220 kb invasion plasmid presents a much smaller target for random insertion of the fusion phage relative to the chromosome, all three classes of temperature-regulated operon fusions were located on the 220 kb virulence plasmid. Detailed mapping of the class 1 mutants placed the fusion end joints within the 37 kb region of the invasion plasmid defined as the minimum sequence required to confer an *Inv*⁺ phenotype on a plasmidless strain of *Shigella* (Maurelli et al., 1986). Possible explanations for the absence of chromosomally located virulence gene fusions could be that virulence genes on the chromosome are: i) not temperature-regulated and therefore would not be detected by the screening method employed; ii) essential genes into which insertions would result in lethal events; or iii) not detectable due to a screening bias in the virulence assay systems used. Conversely, the data reported here suggest that the genes which contribute to expression of the *Inv*⁺, *Pcr*⁺, and *Hly*⁺ phenotypes are the only virulence genes regulated by temperature and that pHS4108 defines the temperature-regulated virulence regulon in *Shigella* spp. However, evidence that *virF* expression is also temperature-regulated (Yoshikawa et al. abstract presented at The 26th Joint Conference U.S.-Japan Cooperative Medical Science Program Cholera and Related Diarrheal Disease Panel, 1990) indicates that the *virR* regulon may include virulence genes located outside of pHS4108. In addition to *virF*, expression of *virB* has also been

shown to be regulated by temperature. These observations again raise the question as to why these genes, known to be required for expression of a full virulence phenotype, were not detected by the screening methods employed in these studies. Mutations in either of these two regulatory genes are not lethal (Sakai *et al.*, 1986a; Sasakawa *et al.*, 1986). Therefore, *virF* and *virB* may fall into a class of temperature-regulated virulence genes which are constitutively expressed. These genes may be expressed at low levels at 30°C and in response to a temperature shift from 30°C to 37°C become further derepressed and increase the pre-existing 30°C levels of expression. Therefore, selection of *Lac*⁻ operon fusion mutants at 30°C would preclude the isolation of *virF* or *virB* fusion mutations.

Six of the insertion mutants isolated fell into two distinct classes (class 1 and class 2) with respect to their effect on the virulence phenotype. Although each of the promoters identified by these two classes of fusions were regulated by *virR*, they differed in the degree to which they were regulated by *virR*. Both the *mxi::lacZ* and *ipaB::lacZ* fusion mutants expressed a 37°C to 30°C β -galactosidase units ratio of >50. In contrast, the *lacZ* fusion mutants which were unaffected in their virulence phenotype, expressed a ratio of <10. Moreover, the levels of β -galactosidase for the *Inv*⁻ fusion mutants at 37°C were higher (>300 units) and the levels at 30°C were lower (<10) than the levels expressed in the *Inv*⁺ temperature-regulated *lacZ* operon fusion mutants (\leq 340 at 37°C and 40-55 units at 30°C). Thus, the *mxi::lacZ* and *ipaB::lacZ* fusion mutants expressed a greater degree of regulation in response to temperature change

with a higher level of transcription of these genes at 37°C and absence of transcription at 30°C. These observations indicate that the temperature-dependent coordinate regulation of virulence genes in *Shigella* may function in such a way as to allow the bacteria to turn virulence genes on to a high level and completely off as needed. The temperature regulation of β -galactosidase in the *Inv*⁺ mutants suggests, however, that virulence genes are not the only genes regulated by temperature. The data indicate that a more subtle regulation may be employed by these non-virulence genes in response to temperature shift from 30 to 37°C. These genes appear to be actively transcribed at both temperatures and the difference in the level of expression between the two temperatures is less pronounced than for the *mxi* genes and the *ipa* operon.

Moreover, the isolation of the *mxi::lacZ* 11.5 fusion promoter adds to a growing list of temperature-regulated virulence gene promoters in *Shigella* which can be further analyzed in regulatory protein-DNA-binding studies. Nucleotide sequence analysis of this promoter may reveal common sequences shared by other genes involved in the temperature-dependent virulence phenotype response which may serve as binding sites for regulatory proteins like *virB*, *virF*, and *virR*. Therefore, this information will contribute to the further elucidation of the molecular mechanisms of temperature-dependent regulation of *Shigella* spp. virulence.

Part II:

Temperature regulation of *Shigella* spp. virulence is dependent on the expression of a chromosomal regulatory locus designated *virR* (Maurelli

and Sansonetti, 1988). The *virR* locus was identified by a mutation which resulted in constitutive expression of a temperature-regulated virulence gene under growth conditions where expression is normally repressed. Therefore, *virR* is a repressor of virulence gene expression at 30°C while at 37°C the virulence phenotype is derepressed. In the second part of these studies, the *virR* locus was further characterized in order to develop an understanding of the molecular mechanisms of *virR* activity.

Identification of a *E. coli* *virR* homolog.

The *Shigella* and EIEC species are human enteroinvasive pathogens which elicit similar disease characteristics and express a temperature-dependent invasion phenotype (Small and Falkow, 1988). Based on the similar virulence phenotypes and close genetic relationship of the *Shigella* and *E. coli* genera, an avirulent laboratory strain of *E. coli* was used to determine if a functionally homologous *E. coli* *virR* gene exists. In these studies, transduction of chromosomal regions from two distinct *E. coli* K-12 strains restored a *VirR*⁺ phenotype in a *virR* mutant strain of *S. flexneri*. The subsequent cloning of the *virR* homolog from C600 clearly demonstrated that *E. coli* K-12 harbored a gene(s) functionally homologous to *virR* from *Shigella*. The *virR* homolog was also confirmed to be located in the same region of the *E. coli* genome (linked to *galU* and *trp*) as it is in *Shigella*. This latter point was not surprising since heteroduplex analysis had revealed that *E. coli* and *Shigella* share >90% overall DNA sequence homology (Brenner et al., 1972) and conjugational gene transfer experiments indicate a chromosomal gene order which is very similar in the two genera (Falkow et al., 1963;

Schneider and Falkow, 1964).

Although the complementation data for the GalU⁺ clone (pAEH001) demonstrated that the clone codes for the *virR* homolog, they deviated somewhat from the expected results. In *Shigella*, *virR* is a transcriptional repressor at 30°C and thus, should only repress the operon fusion promoter at this temperature. The β -galactosidase levels expressed by the BS255/pAEH001 transformants at 37°C, however, were considerably lower than those observed for the parental strain BS255 at 37°C. These results indicate that the clone expressed some repressor activity at 37°C. The disparity between the reported results for the *virR* homolog clone and the wild-type *virR* activity may be explained by the possibility that the cloned DNA represents multiple copies of a gene which normally exists in a single copy. A higher *virR* gene dosage and in turn, an increased concentration of the VirR protein, may have an effect on repression of genes regulated by *virR* even at the normally derepressed temperature. Similar results have been seen previously in the cosmid cloning of the *virR* locus from *S. flexneri* serotype 2a (Maurelli and Sansonetti, 1988) as well as the subcloning of *virR* described below.

The fact that the non-pathogenic *E. coli* K-12 strains harbored a gene capable of virulence gene regulation in *Shigella*, raises questions as to why *E. coli* K-12 would code for such a gene and the evolutionary basis for a non-pathogenic bacteria to maintain a regulator of virulence genes. Several possible explanations for the presence of a *virR* homolog in *E. coli* K-12 exist: 1) the bacteria has lost the genes necessary for expression of a full (*Shigella*-like) virulence phenotype; or 2) *virR* serves as a global regulator of temperature-regulated genes and virulence

genes acquired by the bacteria have adapted the control of their expression to *virR* regulation.

That *virR* may serve as a global temperature-dependent repressor of non-virulence genes is supported by evidence of several temperature-regulated genes in *E. coli* K-12. Genes of the *tra* operon, involved in the conjugative transfer of DNA between bacteria, have been shown to be growth temperature regulated. Studies of *traT* and *traG* as well as the genes responsible for F pilin biosynthesis, demonstrate that expression of their protein products is temperature-regulated (Sowa *et al.*, 1983). The introduction of *virR* mutations into F' strains of *E. coli* followed by analysis of *traT* and *traG*, expression would serve as a simple first step in the determination of whether or not these genes are regulated by *virR*. *envY* regulates the expression of two temperature-regulated major porin proteins, OmpC and OmpF, with no effect however, on the temperature-dependent expression of another major outer membrane protein, OmpT (Lundrigen and Earhart, 1984). Moreover, *envY* does not map to the same region as *virR* nor does cloned *envY* complement a *virR* defect in *Shigella* (A.T. Maurelli, unpublished observations). These results suggest the presence of a temperature-dependent regulator activity which is independent of *envY*. Isolation of *Shigella lacZ* operon fusion mutants in Part I of these studies also suggests that certain temperature-regulated operon fusion mutants, although controlled by *virR*, still exhibit a fully expressed virulence phenotype (Table 3 and 4). The data to be presented below, which indicates that *virR* codes for a protein involved in the structural organization of bacterial chromatin, further substantiates the notion that *Shigella* virulence genes have adapted their expression to

exploit a naturally occurring regulatory system.

All of this evidence lends credence to the possibility that *virR* could play a role as a global temperature-dependent regulator of non-virulence associated operons and genes in *E. coli* as well as in *Shigella*. However, further study will be required before such a conclusion is substantiated.

Characterization of the *Shigella virR* locus.

The *virR* locus was originally isolated from a cosmid library of the *S. flexneri* chromosome (Maurelli and Sansonetti, 1988). To precisely map and identify the *virR* gene, subclones of the 30 kb cosmid insert DNA were isolated and screened for their ability to complement a $\Delta virR$ mutation. Several complementing clones were identified which restored temperature-regulated expression of an *inv::lacZ* operon fusion in the $\Delta virR$ mutant. On the basis of Southern hybridization experiments, in which *virR* complementing clones were used as probes of chromosomal DNA from wild-type and *virR* mutant strains, *virR* was mapped to a single 1.8 kb *EcoRI*-*AccI* fragment (Fig. 10). As shown previously in the present studies and in the earlier *virR* cosmid cloning studies (Maurelli and Sansonetti, 1988), *VirR*⁺ clones characteristically repressed the expression of the reporter gene *lacZ* fusion at both 30 and 37°C (Table 9). Repressor activity detected in these clones at 37°C suggested that multiple copies of *virR* resulted in the activity of a gene product normally undetected at 37°C. The implications of this activity will be discussed in more detail below. Additional complementation analysis revealed that a clone of the *E. coli pilG* locus also expressed a *VirR*⁺ phenotype. *pilG*, a gene required for

the regulation of type 1 piliation in *E. coli* (Spears *et al.*, 1986), maps to 27 minutes on the *E. coli* genetic map and is allelic with *hns* (Kawula *et al.*, manuscript submitted). Comparison of the physical maps of the *pilG* clone, pAL13, and *virR* clone pAEH122, revealed that the insert fragment sizes and restriction maps were identical. The *virR* and *pilG* clones also exhibited a high degree of DNA homology as determined by Southern blot hybridization (Fig. 12). Moreover, nucleotide sequence analysis of a portion of the *virR* clone confirmed the presence of a single large ORF, 412 bp in size, which exhibited over 99% sequence identity with the *E. coli hns* coding sequence. Three base pair differences were detected between the *S. flexneri* and *E. coli* sequences. Two of the nucleotide differences were located outside of the *virR/hns* coding sequence. A single conservative base pair change was observed within the ORF as indicated by the comparison of the predicted amino acid sequences of the *S. flexneri* ORF and the *E. coli hns* coding sequence (Fig. 19). Insertional mutagenesis of the ORF at a unique *HpaI* restriction site abolished $\Delta virR$ complementing activity expressed by the $VirR^+$ clone pAEH122. On the basis of the experimental evidence obtained from the characterization of the $VirR^+$ clone pAEH122, it was concluded that *virR* is an allele of *hns*, a gene which encodes the DNA-binding, histone-like protein H-NS (Pon *et al.*, 1988).

Temperature-dependent regulation of *Shigella* virulence: a model.

The demonstration that *virR* is allelic to *hns* establishes that the temperature-dependent regulator of *Shigella* spp. virulence is a DNA-binding protein thought to be involved in the structural organization of

<i>S. flexneri</i>	1	MSEALKILNNIRTLRAQARECTLETLEEMLEKLEVNVNERREEESAAAAE	50
<i>E. coli</i>	1	50
<i>S. typhimurium</i>	1	50
<i>P. vulgaris</i>	1	...S.....TS.....Q.MQ..	50
<i>S. marcesens</i>	1	...R.....D.Q.Q..	50
<i>S. flexneri</i>	51	VEERTRKLQQYREMLIADGIDPNELNSLAAVKSGTKAKRAQRPAYSYV	100
<i>E. coli</i>	51	100
<i>S. typhimurium</i>	51M..A.....A.....	100
<i>P. vulgaris</i>	51	I...QQ...K...L.....TD..EAAG.S.T. .R...A.....	99
<i>S. marcesens</i>	51	I.....QTM..N.A.G....R.....Q..	100
<i>S. flexneri</i>	101	DENGETKTWTGGRTPAVIKKAMDEQGKSLDDFLIKQ	137
<i>E. coli</i>	101	137
<i>S. typhimurium</i>	101E....Q.....E	137
<i>P. vulgaris</i>	100L...R.IE.E...E....	134
<i>S. marcesens</i>	101IE.....L	134

Figure 19. Comparison of H-NS amino acid sequences from different enterobacterial species. Strains which are compared are shown to the left of each sequence. The complete sequence is that of *S. flexneri* serotype 2a. The other sequences are presented only where they differ from the *S. flexneri* sequence.

the bacterial nucleoid, a structure analogous to the eukaryotic nucleus (reviewed by Drlica, 1987). Among the proteins found in all eukaryotic nuclei are histones, a family of small, basic, and abundant DNA-binding proteins which serve to compact chromosomal DNA into structures referred to as nucleosomes (reviewed by McGhee and Felsenfeld, 1980). In prokaryotes, a group of DNA-binding proteins termed histone-like, based on their shared properties with eukaryotic histones, has been identified (reviewed by Drlica and Rouviere-Yaniv, 1987). Unlike eukaryotic histones however, no evidence exists to indicate that bacterial histone-like proteins (HLPs) form analogous nucleosome structures (Drlica and Rouviere-Yaniv, 1987). Among the DNA-binding proteins characterized as HLPs is H-NS (Lammi *et al.*, 1984) which is believed to correspond to HLPs B1 (Varshavsky *et al.*, 1977) and H1 (Spassky *et al.*, 1984; Hulton *et al.*, 1990). H-NS was initially isolated as one of the major protein components extracted along with *E. coli* chromatin (Lammi *et al.*, 1984). In contrast to other HLPs, H-NS is a neutral protein with an isoelectric point of 7.0 (Lammi *et al.*, 1984). Other properties of the protein, however, are more characteristic of a histone-like protein and include: the relative abundance of H-NS (approx. 20,000 copies/cell), heat stability, a high binding affinity for dsDNA, the ability to compact DNA *in vitro* as well as to increase the thermal stability of DNA (Gualerzi *et al.*, 1986; Friedrich *et al.*, 1988). The primary structure of H-NS consists of 136 amino acids with an M_r of 15 kDa (Falconi *et al.*, 1988) which corresponds to the amino acid sequence predicted from the *hns* structural gene nucleotide sequence (Pon *et al.*, 1988). *hns* maps to 27 minutes on the *E. coli* genetic map and, as alluded to previously, several regulatory loci

(*drdX*, *bglY*, *osmZ*, and *pilG*) which also map to 27 min have been identified and characterized as *hns* genes (Göransson *et al.*, 1990; May *et al.*, 1990; Hulton *et al.*, 1990; Kawula *et al.*, manuscript submitted). Comparison of the *hns* alleles from *E. coli* (Göransson *et al.*, 1990), *S. typhimurium* (Marsh and Hillyard, 1990; Hulton *et al.*, 1990), *S. marsecens*, *P. vulgaris* (LaTeana *et al.*, 1989), and *S. flexneri* reveals that a high degree of nucleotide and predicted amino acid sequence homology exists between the genes isolated from the different bacterial genera (Fig. 19). Therefore, *hns* is a gene highly conserved within the enterobacterial species.

The genetic evidence in this study and in other studies, indicates that *virR/hns* plays a role in the regulation of various unlinked genes and operons. With respect to the temperature-regulation of virulence gene expression in *Shigella*, two fundamental questions remain unanswered: i) what is the molecular mechanism by which VirR/H-NS regulates gene transcription in *Shigella*; and ii) how is temperature change perceived by the organism and integrated into a VirR-dependent global regulatory response?

Based on evidence from a number of studies, a model has been proposed which invokes environmentally-induced alterations in DNA supercoiling as a mechanism of bacterial gene regulation in response to environmental signals (reviewed by Higgins *et al.*, 1990). H-NS is believed to mediate some aspect of the changes in DNA supercoiling. The premise of this model relies on three observations: i) perturbation of DNA supercoiling through mutation or by chemical means results in changes in the expression of various genes; ii) environmental stimuli such as anaerobiosis, osmolarity, and temperature cause changes in DNA super-

coiling; and iii) mutations in *hns* result in altered DNA supercoiling. This model postulates that H-NS alters DNA supercoiling by an undetermined mechanism, and in turn represses gene transcription. Several studies have reported that virulence gene expression in *Salmonella typhimurium* (Galán and Curtiss, 1990) and *S. flexneri* (Dorman et al., 1990) is modified when the bacteria are subjected to conditions which alter DNA supercoiling. All of the evidence presented in favor of this model, however, is indirect since patterns of DNA supercoiling were monitored as variations in reporter plasmid supercoiling as opposed to changes in DNA topology that occur adjacent to the reporter genes studied. Even if one accepts the assumption that reporter plasmid supercoiling reflects an overall change in bacterial DNA supercoiling, these studies highlight certain inconsistencies in the DNA supercoiling model. For example, identical *virR::Tn10* mutations in *E. coli* and *S. flexneri* exhibit the opposite effect on reporter plasmid supercoiling in the two strains (Dorman et al., 1990). Also, the effects of temperature on plasmid DNA supercoiling in *E. coli* are opposite to the effects observed in *S. flexneri* (Dorman et al., 1990). Yet, in the results reported in the present study, the *E. coli* *virR* homolog regulated virulence gene expression in *S. flexneri* in a manner indistinguishable from the *S. flexneri* *virR*. In addition, temperature-regulated expression of *pap* pilin in uropathogenic *E. coli*, which requires the activity of the *hns* allele *drdX* (Göransson et al., 1990), is identical to the pattern of virulence gene regulation in shigellae (Göransson and Uhlin, 1984). Therefore, *E. coli* and *Shigella* spp. regulate virulence gene expression in response to temperature change identically, despite the fact that a mutation in *virR* and temperature

change have different effects on DNA supercoiling in these two genera. Additional problems with the supercoiling model can be found in studies on the regulation of *proU*. The *proU* gene of *E. coli* and *S. typhimurium* was originally demonstrated to be regulated by osmotically induced changes in DNA supercoiling (Higgins *et al.*, 1988). Recently *proU* expression has been shown to be regulated independently of DNA supercoiling (Ramirez and Villarejo, 1991). Thus, rectification of these various experimental discrepancies is necessary before DNA supercoiling can be established as a unifying model for environmentally-regulated gene expression.

Another model which is consistent with the genetic and physiochemical characteristics of *hns* and its gene product can be formulated to answer the questions presented above. H-NS is a DNA-binding protein, and therefore, VirR may bind specific DNA sequences, inhibit RNA polymerase (RNAPol) activity, and thereby repress transcription. Detection and integration of environmental temperature change into a coordinate regulatory response could be achieved by the temperature-regulation of *virR* expression. Based on this rationale, *virR* expression at 30°C would result in synthesis of the repressor which could in turn bind regulatory sequences and repress virulence gene transcription. Conversely, the lack of *virR* expression at 37°C would result in the absence of the regulatory protein and the subsequent derepression of the virulence phenotype. *In vitro* evidence in support of this model shows that H-NS can inhibit the initiation of transcription and *in vivo* evidence suggests that H-NS synthesis is induced when bacteria are shifted from a higher to a lower growth temperature.

In vitro studies corroborate genetic evidence and indicate that H-

NS strongly inhibits DNA transcription (Gualerzi *et al.*, 1986). Studies with H1 show that the protein binds the *lacUV5* promoter and inhibits transcription initiation (Spassky *et al.*, 1984). Although DNase I footprinting analysis of the *lac* and *gal* operon promoters demonstrate that H1 associates with specific DNA-binding protein recognition sites, H1 binding shows no strong sequence specificity (Rimsky and Spassky, 1990). In addition, no *in vivo* evidence exists for H1-mediated regulation of the *lac* operon and, as such, the *lac* operon promoter-operator may not be the optimal sequence to use in the study of H-NS DNA-binding characteristics. On the other hand, the increasing availability of cloned *virR*-regulated genes in *Shigella*, including the *mxl* gene promoter identified in the present studies, provides a potential source of specific VirR DNA-binding sites which could be assessed and compared among the different *virR*-regulated genes.

Identification of H-NS as one of 14 *E. coli* cold shock proteins (Jones *et al.*, 1987; VanBogelen *et al.*, 1990; VanBogelen and Neidhart, 1990) supports the notion that VirR synthesis is temperature-regulated. H-NS synthesis is detected in bacteria grown at 37°C and following a downshift in bacterial growth temperature from 37 to 10°C, the relative rate of H-NS synthesis increases 5-fold over a period of 4.5 h hours. It is conceivable that a reduction in temperature from 37°C to 30°C might also account for a similar, albeit not as dramatic, increase in VirR synthesis. Although observations of H-NS induction during cold shock provide evidence that *virR* expression may increase at 30°C, it is not clear whether the induction of VirR is a result of an increase in transcription, translation, or an increase in the protein's stability at

30°C.

Transcriptional regulation of *hns* in response to changes in environmental temperature has been studied with the *E. coli hns* allele *drdX* (Göransson *et al.*, 1990). These studies demonstrate that the amount of *drdX*-specific mRNA is unaltered when purified from strains grown at either 30 or 37°C. Thus, it would appear that at the transcriptional level, *virR* expression may be unaffected by temperature change. On the other hand, it is possible that only a slight increase in *virR* transcription is required to attain the intracellular VirR concentrations necessary for virulence gene repression at 30°C. Therefore, more sensitive methods to detect *virR* transcripts such as S1 nuclease mapping, primer extension, or polymerase chain reaction transcript amplification may be needed to detect minor changes in mRNA levels. Alternatively, the isolation of *virR::lacZ* operon fusions and measurement of β -galactosidase levels from the fusion construct at 30 and 37°C could serve as a method to measure temperature-dependent *virR* regulation and confirm or disprove previous findings.

If *drdX/virR* is not transcriptionally regulated in response to changes in temperature, then the induction of H-NS synthesis after cold shock may indicate that regulation of VirR synthesis is dependent on temperature-mediated translational regulation. Recent studies have determined that antibiotics which inhibit translation, specifically ribosome function, induce the synthesis of cold-shock proteins (VanBogelen and Neidhart, 1990). Among the cold shock proteins induced was H-NS. Therefore, these studies suggest that induction of VirR synthesis may be the result of an altered translational capacity of the bacteria and that

the ribosome may function to detect changes in environmental temperature. As confirmation of this possibility, *virR::lacZ* protein fusions could be isolated and characterized at 30 and 37°C.

Both the transcriptional and translational regulation of *virR* expression described above invoke the requirement for additional components which regulate expression of *virR*. A plausible explanation for the absence of VirR activity at 37°C which would eliminate this requirement is that the regulatory protein is labile and inactive at 37°C. VirR lability at 37°C might be in the form of a protein conformational change which inhibits DNA-binding activity. The fact that H-NS is described as a heat stable protein which binds nucleic acids *in vitro* at 37°C would appear to discount this possibility (Gualerzi *et al.*, 1986). Evidence from protein cross-linking studies suggests that H-NS can oligomerize to form dimers, trimers, and tetramers (Spassky and Buc, 1977; Gualerzi *et al.*, 1986; Falconi *et al.*, 1988) as H-NS protein concentrations are increased to the predicted intracellular concentration. These data also do not rule out the possibility that the oligomerization of H-NS/VirR may be temperature labile at 37°C. Therefore, specificity of *in vivo* binding to target gene regulatory sequences may depend on oligomer formation which is stable at 30°C. The instability of oligomers at 37°C, on the other hand, would allow for non-specific VirR monomer binding of DNA and of transcriptional regulation. The phenotypes expressed by the VirR⁺ clones, however, indicate that VirR oligomer lability at 37°C is an unlikely explanation for the mechanism by which shigellae detect environmental temperature changes. Each *virR* clone that was isolated represented multiple copies of the regulatory gene and all of these clones repressed

β -galactosidase expression from the reporter gene at both 30 and 37°C (Table 9). In addition, VirR^s clones which exhibit fully repressed β -galactosidase levels at 30 and 37°C could represent higher levels of VirR synthesis than the VirR⁺ clones. Based on these observations, the stability of VirR oligomers, if they are formed, appears not to be temperature labile. Nevertheless, the oligomerization model brings into question the conditions under which VirR DNA binding studies are conducted and suggests that alternative conditions, ones which mimic *in vivo* conditions at 30°C, may be necessary to determine the true regulatory mechanisms of VirR.

All of the *virR* regulatory scenarios presented above describe several alternatives for the response of shigellae to alterations in environmental temperature and translate this change into a global regulatory response. The identification of H-NS as a cold shock protein capable of oligomerization, along with evidence that ribosomes may serve as the cellular components which monitor temperature change (VanBogelen *et al.*, 1990), provide for the formulation of a model of VirR-dependent virulence gene regulation presented in Figure 20. Transcription of *virR* would be unaffected by a decrease in temperature from 37 to 30°C as suggested by the characterization of *drdX* transcription (Göransson *et al.*, 1990). However, the translation of the *virR* transcript would increase at 30°C, due to increased ribosomal activity and result in increasing concentrations of VirR monomer. An increased intracellular pool of VirR monomer would then promote a concentration dependent VirR oligomerization, as suggested by H-NS crosslinking experiments (Spassky and Buc, 1977; Gualerzi *et al.*, 1986; Falconi *et al.*, 1988). As mentioned

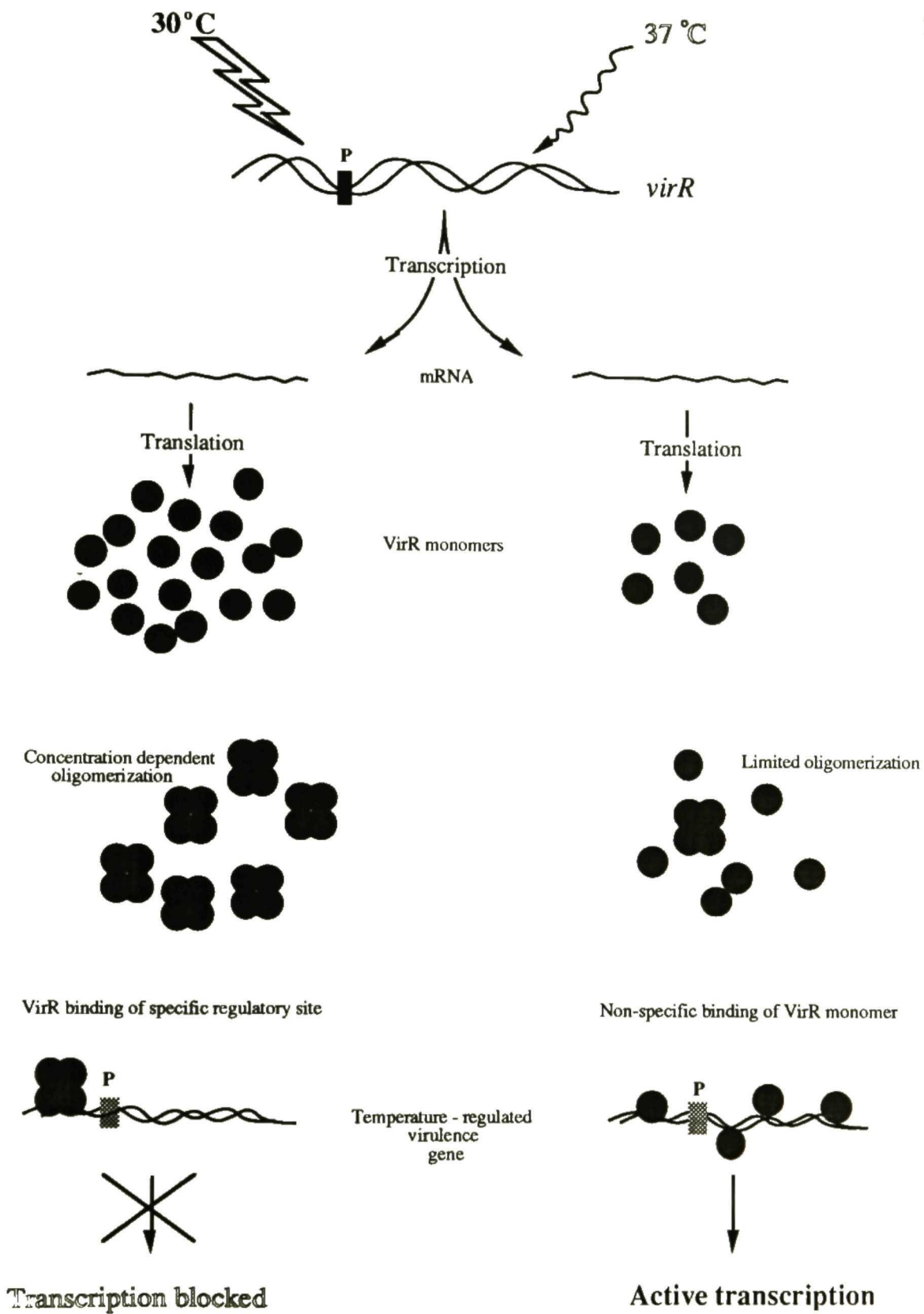


Figure 20. Proposed mechanism of VirR dependent virulence gene regulation in *Shigella* spp.

above, the formation of oligomers could facilitate DNA sequence specific binding of VirR and thus inhibit virulence gene transcription. At 37°C, lower levels of translation would result in lower levels of VirR monomer synthesis and thus preclude oligomer formation. Although monomers could non-specifically bind to DNA under these conditions, transcription would be unaffected. Concentration dependent oligomerization is also consistent with the results presented in the present studies. VirR⁺ clones which represent increased *virR* transcript synthesis could account for a sufficient concentration of VirR monomer to lead to increased oligomer formation.

Complementation of $\Delta virR$ and regulation of virulence gene expression by tRNA^{Tyr}.

Among the $\Delta virR$ complementing clones isolated, pAEH104 exhibited a VirR^P phenotype. In contrast to the VirR⁺ phenotype expressed by *virR* clone pAEH122, pAEH104 did not repress virulence gene transcription in the $\Delta virR$ mutant at 30°C to wild-type levels (Table 9). All of the VirR⁺ clones reduced the reporter strain β -galactosidase levels to <10 units, whereas pAEH104 reporter strain transformants consistently expressed 3-fold higher levels of enzyme at 30°C. Restriction site mapping and Southern blot hybridization indicated that the 2.3 kb *EcoRI*-*AccI* pAEH104 insert fragment was distinct from the 1.8 kb *EcoRI*-*AccI* insert in *virR* clone pAEH122 (Fig. 11). The VirR^P activity was also shown not to be limited to the *inv::lacZ* operon fusion reporter system used. Both a *mxi::lacZ* fusion and *IpaB*, *C*, and *D* expression were also partially regulated by the pAEH104 subclone (Table 10 and Fig. 14). Determination

of the entire 2.3 kb fragment nucleotide sequence and comparison of the sequence to other known bacterial sequences revealed a high degree of nucleotide sequence homology to the *tyrT* locus of *E. coli* K-12 (Fig. 16; Goodman *et al.*, 1968; Sekiya *et al.*, 1976). Unlike the *tyrT* locus from *E. coli*, the *tyrT* locus from *S. flexneri* contained only the wild-type $\text{tRNA}_1^{\text{Tyr}}$ coding sequence and its native promoter (Fig. 17). Sequence identity for either a second copy $\text{tRNA}_1^{\text{Tyr}}$ or the *E. coli* protamine-like protein also known to map to the *tyrT* locus (Altman *et al.*, 1981) was not detected. Similarly, the three 178 bp repeat sequences found in the *E. coli* locus (Egan and Landy, 1978) were absent in *S. flexneri*. Subclones of the 2.3 kb *EcoRI*-*AccI* fragment used to map the Vir^P activity revealed that deletion of either a part of or the entire $\text{tRNA}_1^{\text{Tyr}}$ sequence resulted in loss of the Vir^P phenotype (Fig. 18). Furthermore, convergent transcription of the $\text{tRNA}_1^{\text{Tyr}}$ promoter with the P_{lac} promoter resulted in a loss of VirR^P activity (data not shown). A single clone, pAEH123, which contained only the $\text{tRNA}_1^{\text{Tyr}}$ coding sequence confirmed that the presence of $\text{tRNA}_1^{\text{Tyr}}$ expressed in multi-copy was capable of suppressing a ΔvirR mutation in *Shigella* (Fig. 18).

That an essential component of bacterial translation is able to regulate gene expression at the transcriptional level is not unprecedented. Both transcription attenuation and the stringent response are situations in which bacterial transcription is modulated by the availability of amino acylated or charged tRNA molecules to incorporate amino acids into a newly synthesized polypeptide. Based on the multi-copy nature of the $\text{tRNA}_1^{\text{Tyr}}$ represented by pAEH104 it was possible that a large proportion of molecules were uncharged. An imbalance in the ratio

of charged vs uncharged tRNA₁^{Tyr} might therefore, result in the VirR^P activity exhibited by tRNA₁^{Tyr} clones. Another possible explanation for the expression of the VirR^P phenotype is that the tRNA molecule facilitates the DNA-binding of a mutant VirR protein in the reporter strain.

Transcription attenuation (reviewed by Landick and Yanofsky, 1987) involves the potential formation of secondary structures in mRNA leader sequences, referred to as attenuator sites, which when formed, can cause transcription termination. The leader peptides of amino acid biosynthetic operons which are regulated by attenuation, characteristically contain multiple residues of the amino acid synthesized by the operon arranged in succession. Incorporation of these amino acids during leader peptide synthesis is dependent on the intracellular levels of charged cognate tRNAs and provides the basis for determination of whether or not an mRNA terminator structure will be formed at the attenuator. Under conditions where amino acylated tRNA concentrations are limiting, leader polypeptide synthesis is stalled, an mRNA antiterminator is formed and transcription proceeds uninhibited. A similar stalling of polypeptide translation is thought to induce the stringent response. The stringent response is generally defined as a set of complex physiological changes that occur in *E. coli* when amino-acylated tRNA become the limiting factor in protein synthesis (reviewed by Cashel and Rudd, 1987). Induction of the stringent response includes a reduction in total RNA synthesis along with the increased synthesis of guanosine tetraphosphate (ppGpp). ppGpp is implicated as the pleiotropic regulator of gene expression during the stringent response. Thus, during translation, if polypeptide synthesis is inhibited due to a lack of sufficient charged tRNA, ppGpp levels

increase and transcription is repressed.

Based on the transcriptional attenuation models developed for the various amino acid biosynthetic operons, if tyrosine is required in the translation of *virR*-regulated genes, a decrease in the charged to uncharged tRNA^{Tyr} ratio would actually enhance transcription of these genes. Therefore, the predicted effect of the tRNA^{Tyr} clone, pAEH104, on the expression of the reporter *lacZ* operon fusion in BS211 would be the converse of the results that were observed (Table 9). In addition, the predicted amino acid sequences of genes which may come under the direct control of *virR* such as *virF* (Sasakawa *et al.*, 1986), *virB* (Adler *et al.*, 1989), and the *ipa* operon (Baudry *et al.*, 1988; Venkatesan *et al.*, 1988; Sasakawa *et al.*, 1989), indicate that no strings of tyrosine residues are found in the amino-terminal portions of these protein sequences. Thus, it appears unlikely that a transcription attenuation mechanism is responsible for the VirR^P phenotype expressed by tRNA^{Tyr} cloned in pAEH104.

Studies which have examined the effects of tRNA over-production on ppGpp synthesis demonstrate that ppGpp can be induced in bacteria grown under amino acid limiting conditions when the ratio of charged to uncharged tRNA ranges from 0.1 to 0.2 (Rojiani *et al.*, 1989). tRNA^{Tyr} clone transformants of *Shigella* may represent a charged/uncharged tRNA^{Tyr} ratio which triggers a stringent response. Increased ppGpp synthesis would, in turn, repress virulence gene transcription. Evidence from recent studies which examined the pleiotropic effects of tRNA^{Leu} overproduction in *E. coli* would appear to contradict this reasoning (Wahab *et al.*, 1991 manuscript submitted). In these studies, a clone of the tRNA^{Leu} operon which contains three copies of the tRNA₁^{Leu} coding sequence, causes a 15-fold increase in

intracellular concentrations of tRNA₁^{Leu} when transformed into *E. coli*. Moreover, under conditions where only 40% of the tRNA₁^{Leu} were charged, increased intracellular ppGpp levels are not detected. Therefore, the tRNA^{Tyr} clone pAEH104 which represents a single tRNA^{Tyr} coding sequence in multiple copy is unlikely to have caused an induction of the stringent response in the $\Delta virR$ mutant. Furthermore, induction of both transcriptional attenuation and the stringent response by decreasing the level of charged tRNA is accomplished by exposing the bacteria to growth conditions where concentrations of a specific amino acid are limiting. The use of rich media to culture *Shigella* prior to assay for the expression of the temperature-regulated phenotype in these studies, suggests that the level of tyrosine necessary to charge the tRNA molecules is not limiting. Nevertheless, neither transcriptional attenuation nor the stringent response serve to explain the temperature-dependent complementation observed for pAEH104. In either situation, one would expect that a complete repression of transcriptional activity would be observed at both 30 and 37°C.

As alluded to above, another possible explanation for the complementation phenotype observed for the tRNA₁^{Tyr} clones is that the tRNA molecule may physically bind to a truncated VirR and facilitate expression of repressor activity in the $\Delta virR$ mutant. As yet, the endpoints of the mutation have not been defined and the deletion may result in the expression of a truncated protein incapable of oligomerization. *In vitro*, H-NS does bind to tRNA (Gualerzi et al., 1986). In addition, tRNA molecules exhibit a high degree of secondary structure and formation of hairpin loops which may serve as nucleation sites of other nucleic acids

or proteins. Examples of regulatory proteins binding specific RNA stem loop structures have been reported in both *E. coli* RNA bacteriophages Q β (Witherell and Uhlenbeck, 1989) and R17 (Carey *et al.*, 1983) as well as in human immunodeficiency virus (Cheong *et al.*, 1990). An analogous interaction of tRNA_{1^{Tyr}} with VirR therefore may have occurred. On the basis of the results presented in these studies, tRNA_{1^{Tyr}} expressed in high copy functions is a novel mechanism which complements a Δ virR mutation in *S. flexneri*.

Summary.

The primary objective of the studies presented here was to expand the current knowledge of the temperature-regulation of virulence gene expression in *Shigella* spp. The approach taken to address this regulatory phenomenon was two fold. On the one hand new virulence genes which were regulated in response to temperature were identified and characterized. On the other hand the temperature-dependent virulence gene regulator, virR, was isolated and characterized. On the basis of the results presented here, at least two new temperature-regulated virulence genes, designated *mxl* were characterized as being involved in the surface expression of other known invasion associated proteins. Furthermore, one of the *mxl* temperature-regulated promoters was isolated. The virulence gene regulator virR was identified as an allele of the histone-like cold shock protein gene *hns*. In addition, identification of a virR homolog in non-pathogenic strains of *E. coli* suggested that virR may also function as a temperature-dependent regulator of virulence and non-virulence genes. The identification of the virR coding sequence along with the isolation

of the temperature-regulated *mxi* loci should facilitate and enhance further genetic and biochemical analysis of temperature-dependent virulence gene regulation in the *Shigella* spp.

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